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THE FUNCTIONS OF THE CORPUS ALLATUM IN *RHODNIUS PROLIXUS* (HEMIPTERA)

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(With Plate 1 and Four Text-figures)

It has been shown in earlier papers (Wigglesworth, 1934, 1936, 1940) that metamorphosis in *Rhodnius* takes place when the corpus allatum ceases to secrete the 'inhibitory' or 'juvenile hormone'. There was no evidence for the existence in this insect of a 'metamorphosis hormone' which promotes the development of imaginal characters. Growth and moulting are induced at all stages of development by a 'moulting hormone' (what is called by Scharrer & Scharrer (1944) a 'growth and differentiation hormone') secreted in the dorsum of the protocerebrum. When this hormone alone is acting, the latent imaginal characters are realized and metamorphosis occurs. When the 'juvenile hormone' from the corpus allatum of the early nymphal stages is present in addition, it causes the preferential development of nymphal characters and thereby suppresses those of the adult.

This conception of growth regulation in insects has proved applicable to Orthoptera (*Dixippus* (Pflugfelder, 1937, 1939); *Melanoplus* (Pfeiffer, 1945*a*); *Leucophaea* (Scharrer, 1946)); to Lepidoptera (*Bombyx*, *Lymantria*, *Galleria* (Bounhiol, 1938); *Ephestia*, *Galleria* (Piepho, 1940, 1942)); and to Coleoptera (*Tenebrio* (Radtke, 1942)). In the Diptera, on the other hand, the 'ring gland', which is a composite structure with elements representing the corpus allatum, the corpus cardiacum and perhaps the 'pericardial gland' and the hypocerebral ganglion (Poulson, 1945; Thomsen, 1942; Vogt, 1943*a*), appears actively to induce metamorphosis as though it were secreting a metamorphosis-promoting hormone (Hadorn, 1941).

There is some evidence, however, that the central part of the ring gland, the part representing the corpus allatum, restrains the differentiation of the imaginal disks in the young larva of *Drosophila* (Vogt, 1943*a*), and it has recently been shown by Vogt (1946) that the corpus allatum of the adult *Drosophila* implanted into the larva will cause a local suppression of adult characters in the resulting fly.

These findings go some way towards bringing the control of metamorphosis in the Diptera into line with that in other insects. It is the object of the present work to see whether the corpus allatum in *Rhodnius*, besides ceasing to produce the juvenile hormone, also plays an *active* part in the causation of metamorphosis.

FUNCTION OF THE CORPUS ALLATUM DURING MOULTING IN THE 5TH-STAGE NYMPH

The secretion of the juvenile hormone by the corpus allatum ceases when the 4th-stage nymph has moulted to the 5th stage (Wigglesworth, 1936). But during the moulting of the 5th-stage nymph to the adult, the corpus allatum again shows signs of renewed activity, the cytoplasm of the cells becoming swollen and dense. At first this was taken as evidence that the gland was secreting the moulting hormone (Wigglesworth, 1934); but since that was disproved and it was shown that the moulting hormone comes from the dorsum of the brain (Wigglesworth, 1940) the meaning of this renewed physiological activity has been obscure.

The question has been studied by comparing the structural characters produced by 5th-stage nymphs moulting in the presence and absence of the corpus allatum. This has been effected in three ways:

(i) 5th-stage nymphs were allowed to develop for 9 days after feeding, by which time they have, for the most part, passed the critical period and can moult after decapitation (Wigglesworth, 1934). Half were decapitated completely with removal of the corpus allatum; half had the head cut through behind the brain but in front of the corpus allatum.

Both groups developed adult characters. No differences could be seen in the structure of the abdominal cuticle. In both, the unexpanded wings were highly crumpled; but here a difference was detectable, the wings being more deeply folded in the second group. Likewise in the genitalia, the adult characters were developed more completely in the second group of insects, those which retained the corpus allatum.

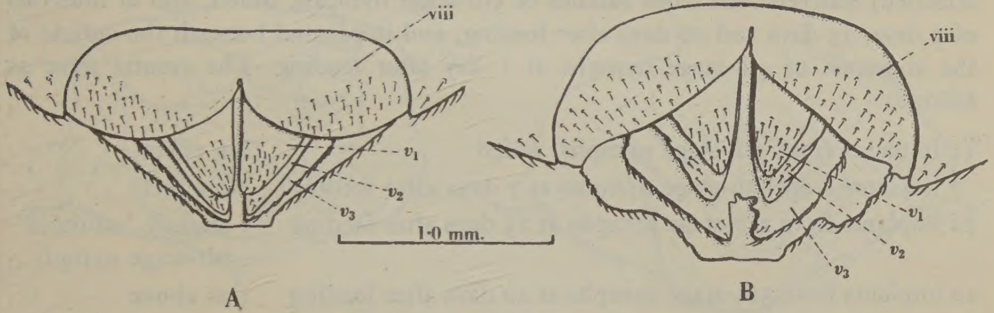
(ii) In the above experiment the corpus allatum was retained in both groups of insects until after the critical period, and may be supposed to have exerted some action by this time. 5th-stage nymphs were therefore decapitated 24 hr. after feeding and were then induced to moult by joining them, as already described (Wigglesworth, 1934), to 5th-stage nymphs decapitated at 10 days after feeding. In six experiments both insects were deprived of their corpus allatum; in six the corpus allatum was retained in both.

The results were more striking than in the former series. The wings were not entirely normal even in the insects which retained the corpus allatum, the folding being slightly less extreme than in a normal adult insect (Pl. 1, fig. 1). But in the insects without the corpus allatum, the wings were in part definitely nymphal. There was considerable individual variation, but in several, although there was some folding towards the base, the apical half was more or less smooth, as in the nymph (Pl. 1, fig. 2).

There were differences also in the cuticle of the abdomen. In those with the corpus allatum, the 'hinge-line' was fully formed, the pigmentation was of the normal adult type (as represented in Wigglesworth, 1940, Pl. I, fig. 8) and the folding of the epicuticle was shallow and transverse as in the adult. In those without the corpus allatum the 'hinge-line' was incompletely formed over the middle and anterior abdominal segments, the pigment spots had a partially nymphal distribution tending to extend towards the postero-lateral angles of the segments, and over much of the abdomen the epicuticular folds showed some change towards the stellate type characteristic of the nymph. The whole appearance resembled that shown in Wigglesworth (1940, Pl. I, fig. 6), which represented an insect that had been switched over from adult development to nymphal development on the thirteenth day after the commencement of moulting.

(iii) In the third series, moulting in the complete absence of the corpus allatum was secured by decapitating a number of 5th-stage nymphs at 24 hr. after feeding and implanting into the abdomen the dorsum of the brain from other 5th-stage nymphs which had been fed 12 days previously.

The results were like those obtained in the preceding series. The characters in the main were those of the adult. But the wing lobes, the genitalia (Text-fig. 1 B), and the abdominal cuticle still showed vestiges of nymphal characteristics.



Text-fig. 1. A, external genitalia of normal adult female; B, genitalia of an adult female decapitated in 5th stage 1 day after feeding and caused to moult by the implantation of another 5th-stage brain without the corpus allatum. viii, eighth sternite; v_1, v_2 and v_3, the first, second and third pairs of valvulae.

The second type of experiment (series (ii) above) was repeated with 4th-stage nymphs decapitated at 24 hr. after feeding and joined to 5th-stage nymphs which had passed the critical period, with and without the corpus allatum. The results were not so striking as with the 5th-stage nymphs. Both groups of 4th-stage nymphs developed adult characters, but once more these were better formed, notably in the folding of the wings, when the 5th-stage nymphs retained the corpus allatum.

It is clear from these results that the corpus allatum of the 5th-stage nymph in *Rhodnius* is playing at least a small active part in the completion of metamorphosis. There are two ways in which it might be supposed to act:

(*a*) By the secretion of a 'metamorphosis hormone' which acts on the tissues and favours the production of adult characters (as the 'juvenile hormone' favours the production of nymphal characters).

(*b*) By the active elimination of juvenile hormone persisting in the blood and tissues.

An attempt was made to differentiate between these alternatives by implanting the corpus allatum from the 5th-stage nymph at varying stages in its development, into the abdomen of 4th-stage and 3rd-stage nymphs. In these experiments the host insect in the 3rd or 4th stage retains its own corpus allatum, which we know is secreting the juvenile hormone and so preventing metamorphosis. We know also that the corpus allatum of the 3rd- and 4th-stage nymph, implanted into the abdomen of a 5th-stage nymph, suppresses metamorphosis and leads to the production of a 6th-stage nymph. The corpus allatum from the 5th-stage nymph could scarcely be expected, therefore, to produce a very striking effect when implanted into the 3rd- or 4th-stage nymph. But if it does indeed secrete a 'metamorphosis hormone' we might expect to find developed a small patch of imaginal cuticle in immediate proximity to the implanted gland—just as implants of the young corpus allatum into the 5th-stage nymph, if they fail to lead to the production of 6th-stage nymphs, often produce adults with a small area of nymphal cuticle overlying the implant.

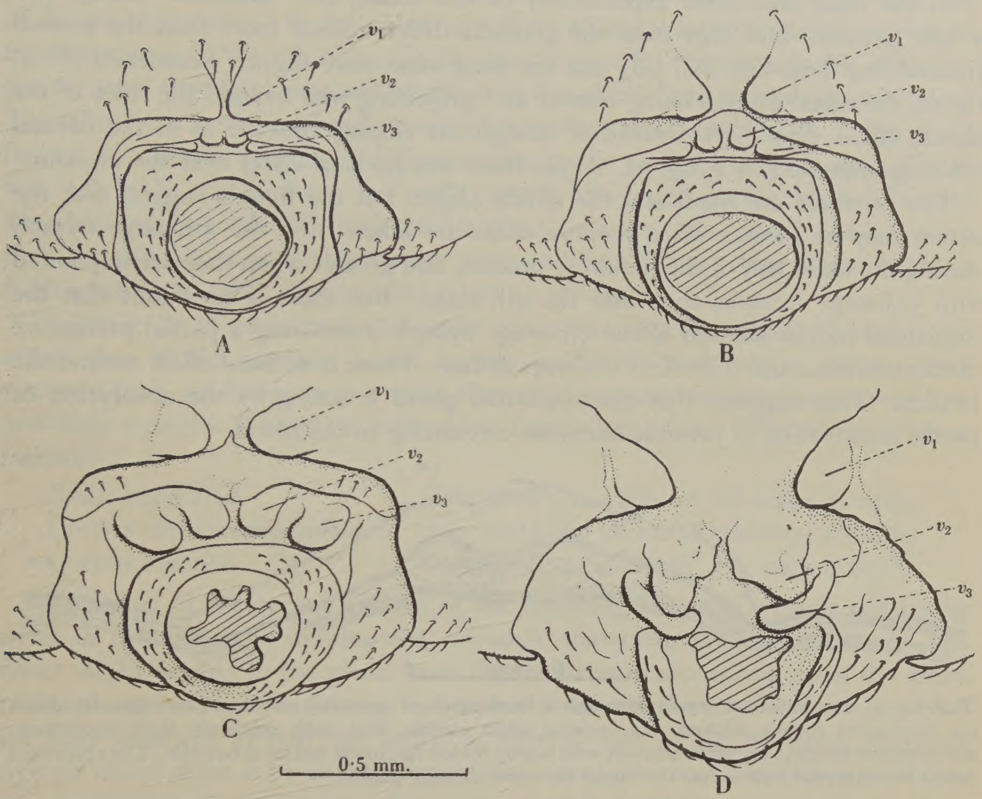
The corpus allatum (together with the corpus cardiacum to which it is closely attached) was removed from batches of 5th-stage nymphs, unfed, and at intervals of 7 days, 15 days and 20 days after feeding, and implanted beneath the cuticle of the abdomen of 4th-stage nymphs at 1 day after feeding. The results were as follows:

11 implants from 5th-stage nymphs, unfed	No effect
7 implants from 5th-stage nymphs at 7 days after feeding	No effect
12 implants from 5th-stage nymphs at 15 days after feeding	1 slightly 'adultoid' 5th-stage nymph
12 implants from 5th-stage nymphs at 20 days after feeding	1 as above

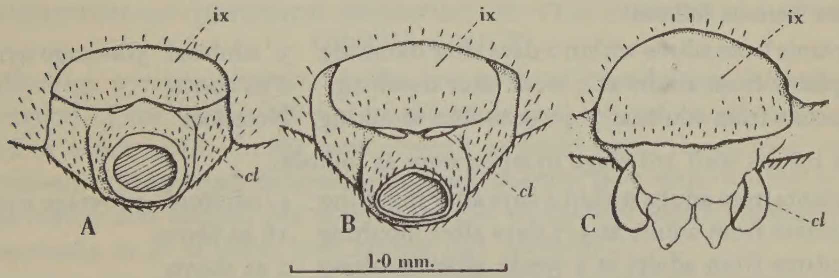
The term 'adultoid' nymph is adopted after Scharrer (1946) to describe insects in which the differentiation of imaginal characters has proceeded further than in the normal nymph. In these two instances the effect was very small; there was no detectable change in the wing lobes, but the first pair of valvulae (on the 8th abdominal segment) in the female was a trifle more widely separated than normally (cf. Text-fig. 2B). There was no effect on the abdominal cuticle overlying the implant.

In the foregoing experiment the newly implanted corpus allatum, without an established tracheal supply, is competing with the undamaged corpus allatum of the host. It seemed possible that a more striking effect might be produced if the corpus allatum from the 5th-stage nymph were implanted into the 3rd-stage nymph so as to give it an opportunity to become well established by the time the 4th-stage nymph moulted. In none of these insects was there any detectable effect on the characters of the resulting 4th-stage nymphs. The effects on the resulting 5th-stage nymphs were as follows:

15 implants from 5th-stage nymphs, unfed	1 slightly 'adultoid' 5th-stage nymph
15 implants from 5th-stage nymphs at 7–10 days after feeding	1 as above
10 implants from 5th-stage nymphs at 15 days after feeding	1 as above
11 implants from 5th-stage nymphs at 20 days after feeding	3 as above



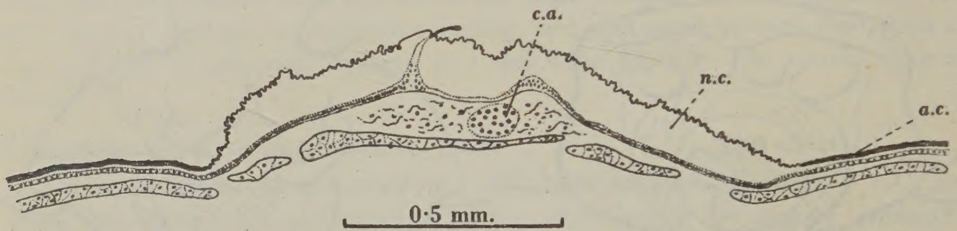
Text-fig. 2. A, female genitalia of normal 5th-stage nymph; B, the same in slightly 'adultoid' 5th-stage nymph produced by the implantation of the corpus allatum of 5th-stage nymph 15 days after feeding into a 4th-stage nymph; C, the same in moderately 'adultoid' 5th-stage nymph produced by implanting corpus allatum of adult 1 week after moulting into a 3rd-stage nymph; D, the same in strongly 'adultoid' 5th-stage nymph produced as C. v_1, v_2 and v_3, the first, second and third pairs of valvulae.



Text-fig. 3. A, male genitalia of normal 5th-stage nymph; B, the same in slightly 'adultoid' 5th-stage nymph produced by the implantation of the corpus allatum of an adult 2 days after moulting into a 2nd-stage nymph; C, the same in a strongly 'adultoid' 5th-stage nymph produced by implantation of corpus allatum of a newly moulted adult into a 3rd-stage nymph. ix, ninth sternite; *cl*, rudiments of claspers.

In the most successful experiments of this series, the 'adultoid' change was a little greater. Not only were the genitalia differentiated more than the normal (resembling Text-figs. 2C, 3B), but the wing lobes were slightly broadened (Pl. 1, fig. 6), the costal margin being convex and projecting well beyond the sides of the thorax (Pl. 1, figs. 6, 7), instead of straight or slightly concave as in the normal 5th-stage nymph (Pl. 1, fig. 3). Again there was no local effect over the implant.

The numbers are small and the effects slight, but the figures suggest that the action may be greater when corpora allata are taken from the 5th-stage nymph during the latter half of the moulting process, and greater when they are implanted into 3rd-stage nymphs than into the 4th stage. But there is no doubt that the implanted corpus allatum of the 5th-stage nymph is inducing a partial premature metamorphosis, and this effect is always diffuse. There is no local effect around the implant. That suggests that the implanted gland is acting by the absorption or partial inactivation of juvenile hormone circulating in the blood.



Text-fig. 4. Longitudinal section through a local patch of nymphal cuticle in an adult *Rhodnius*. *c.a.* implanted corpus allatum; *a.c.* normal adult cuticle, thin with relatively thick exocuticle; *n.c.* nymphal cuticle, very thick and soft with highly folded epicuticle but no exocuticle. The epidermis below the nymphal cuticle has the nuclei far more densely packed.

EFFECT OF THE CORPUS ALLATUM OF THE ADULT ON THE DIFFERENTIATION OF ADULT CHARACTERS

It seemed possible that the corpus allatum of the young adult insect might continue to exert this same action of eliminating the juvenile hormone. This was tested by implanting the gland (together with the corpus cardiacum as before) from adults of known age into 3rd-stage and 4th-stage nymphs. The results with 4th-stage nymphs were as follows:

13 implants from adults within 2 days after moulting	3 'adultoid' 5th-stage nymphs
15 implants from adults at 1 week after moulting	2 as above
8 implants from adults at 3–4 weeks after moulting	No effect

The results with 3rd-stage nymphs were as follows:

13 implants from adults within 2 days after moulting	4 'adultoid' 5th-stage nymphs
30 implants from adults at 5–7 days after moulting	16 as above
9 implants from adults at 2 weeks after moulting	1 as above
12 implants from adults at 3–4 weeks after moulting	No effect

A few implants were made into 2nd-stage nymphs with results as follows:

12 implants from adults about 5 days after moulting	3 slightly 'adultoid' 5th-stage nymphs

The corpus allatum of the newly moulted adult clearly has the same effect as that of the 5th-stage nymph in the later stages of moulting; it causes a partial premature metamorphosis in the 5th stage. In the most successful experiments the effect was greater than that following implantation of the gland from the 5th-stage nymph. The wing lobes were broad and crumpled or swollen and distended (Pl. 1, figs. 8, 9); there was a greater degree of differentiation in the genitalia of both sexes (Text-figs. 2D, 3C), and in some the ocelli (which occur only in the adult *Rhodnius*) were becoming visible. The numbers involved are again rather small, but it appears that this effect is exerted only by the corpus allatum of the young adult; implants from adults 3–4 weeks after moulting had no action.

In one series of experiments, in order to be sure that the corpus allatum is the active part of the implant, brain, corpus cardiacum and corpus allatum were removed from adult insects at 1 week after moulting and implanted separately into 3rd-stage nymphs. The parts were taken from eight adults. The results were as follows:

Brain implanted	6 survived; no effect on 5th-stage nymphs.
Corpus cardiacum implanted	7 survived; no effect on 5th-stage nymphs.
Corpus allatum	8 survived; 5 'adultoid' 5th-stage nymphs.

The action of the corpus allatum of the moulting 5th-stage nymph and young adult in favouring the development of adult characters is thus confirmed. The effect might be due to the gland from the maturing insect acting upon the gland of the young host and weakening its capacity to secrete the juvenile hormone. But we have seen (p. 2) that the action is exerted also in the moulting 5th-stage nymph where there is no young corpus allatum to influence. The failure to obtain in a single experiment any local formation of adult characters around the implant, makes it unlikely that the effect is due to a 'metamorphosis hormone' favouring imaginal differentiation directly. It seems more probable that the gland is either absorbing the juvenile hormone circulating in the blood and so withholding it from its site of action (and that is perhaps the most likely explanation) or it may be producing a secretion which inactivates the juvenile hormone that is in circulation.

SECRETION OF JUVENILE HORMONE BY THE CORPUS ALLATUM OF THE ADULT

The 5th-stage nymphs produced in the last series of experiments, following the implantation of the corpus allatum of the adult into the 3rd or 4th stage, were allowed to moult again. In the most strongly adultoid forms the ecdysial line on the head and thorax had disappeared. Consequently they were unable to split the old cuticle and escape; but apart from this mechanical defect, moulting took place normally in all of them, both normal and 'adultoid'.

The characters of the resulting adults were either normal or 'nymphoid'. In some there was only a small patch of nymphal cuticle immediately overlying the implant (Pl. 1, fig. 5); in others the entire character of wings, cuticle and genitalia might be intermediate between nymph and adult (Pl. 1, fig. 10); others were completely nymphal—6th-stage nymphs (cf. Pl. 1, fig. 12).

Where there was a localized patch of nymphal cuticle over the implant, it could be seen in sections (Text-fig. 4) and dissections that the corpus allatum, richly supplied by tracheae running in from all sides, was rounded and well defined and lay exactly below the centre of the nymphal patch. In some instances the patch was so minute (100μ in diameter) that it scarcely exceeded the area of the underlying gland.

Clearly the implanted corpus allatum, derived from an adult insect, is now secreting juvenile hormone. A similar result was obtained by Pfeiffer (1945*a*) in *Melanoplus*. When the corpora allata from the adult of this grasshopper were implanted into the 5th-stage nymph, the resulting 7th stage instead of being an adult was partially or wholly nymphal; and these insects might moult again to produce an 8th or even a 9th stage.

Now we know that the imaginal tissues, exposed to the combined action of the moulting hormone and the juvenile hormone of the young nymphal stages will revert, in part at least, to the nymphal state (Wigglesworth, 1940). The question arises, therefore, whether the juvenile hormone is a normal secretion of the corpus allatum of the adult; or whether it is produced only when the gland has become 'rejuvenated' by exposure to the secretion of the 3rd- or 4th-stage nymph.

This has been tested in two ways:

(i) By the implantation of the corpus allatum from adults of known age directly into 5th-stage nymphs at 24 hr. after feeding. The results were as follows:

10 implants from newly moulted adults produced:
1 6th-stage nymph; 1 nymphoid adult; 5 adults with a small patch of nymphal cuticle over the implant; and 3 normal adults.

10 implants from adults 3 weeks old produced:
5 6th-stage nymphs; 2 nymphoid adults; 3 adults with distinct nymphal patches; and 1 normal adult.

Thus the juvenile hormone is being secreted by the corpus allatum removed from the adult without its having been first rejuvenated in the early nymph. Moreover, the production of the hormone would appear to be more active in the mature adult than in the newly moulted insect. This same difference is apparent when implantations are made into the 3rd- or 4th-stage nymph:

13 implants from newly moulted adults into 3rd-stage nymphs gave rise finally to:
1 nymphoid adult; 5 adults with small patches of nymphal cuticle; 7 normal adults.

10 implants from adults 3 weeks old into 3rd-stage nymphs gave rise finally to:
1 6th-stage nymph; 1 nymphoid adult; 6 adults with well-marked patches of nymphal cuticle; 2 normal adults.

7 implants from newly moulted adults into 4th-stage nymphs produced:
3 adults with very minute local patches of nymphal cuticle; 4 normal adults.

6 implants from adults 3 weeks old into 4th-stage nymphs produced:
6 adults with well-marked nymphal patches.

(ii) The second procedure was to allow 5th-stage nymphs to pass the critical period; then to decapitate them at 10 days after feeding with removal of the corpus allatum, and to join them to mature adults (about 3 weeks old; fed 1 day previously) in which the anterior part of the head with the brain were removed but the corpus allatum retained. The moulting 5th-stage nymph was thus deprived of its own corpus allatum and exposed to the secretion of the intact corpus allatum of the adult. And the adult was exposed to the moulting hormone of the 5th-stage nymph and to the secretion of its own corpus allatum.

The 5th-stage nymphs when they moulted developed characters in the wing lobes and in the cuticle of the abdomen that were definitely nymphal. In six experiments out of eight in which the 5th-stage nymphs moulted, the adult also was caused to moult. And in those experiments in which the 5th-stage nymph retained its nymphal characters most distinctly, the new cuticle produced by the adult showed a partial reversion to the nymphal condition. This effect was not so pronounced as in the best examples resulting from the implantation of young corpora allata into the adult (Wigglesworth, 1940, Text-figs. 10, 11), but it was unmistakable. There were areas on the abdomen where the new epicuticle was thrown into folds intermediate in type between those of nymph and adult.

The juvenile hormone must therefore be a normal secretion in the mature adult. Thus the corpus allatum in the adult appears to have a dual function. (*a*) In the early days after moulting it absorbs or inactivates the juvenile hormone; this function is carried over apparently from the moulting 5th-stage nymph, and is lost in a week or two after the insect becomes adult. (*b*) It begins to secrete again the juvenile hormone; this activity appears to be slight in the newly moulted adult but to increase markedly in the mature insect.

FUNCTION OF THE JUVENILE HORMONE IN THE NORMAL ADULT

The juvenile hormone is apparently produced in increasing quantity in the adult *Rhodnius* at the same time as the 'gonadotropic hormone', responsible for the formation of yolk in the female and for the activity of the accessory glands in the male, is being secreted by the corpus allatum. Pfeiffer (1945*a*) indeed, has suggested that in *Melanoplus* the 'gonadotropic hormone' of the adult may be identical with the juvenile hormone of the young nymph.

If that is so, not only should the secretion from the adult restrain metamorphosis in the nymph and induce a reversal of metamorphosis in the moulting adult (as described above), but the secretion from the corpus allatum of the young nymph should induce egg formation in the adult female.*

It was shown in an earlier paper (Wigglesworth, 1936) that the decapitated adult female deprived of its corpus allatum will not develop eggs. If joined to an adult of either sex which retains its corpus allatum, eggs are developed. If joined to a moulting 5th-stage nymph retaining its corpus allatum no egg development occurs—indeed the adult may be induced to moult by this means. It has now been found that the secretion from the corpus allatum of the moulting 4th-stage nymph will induce egg formation in the decapitated adult female.

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* Vogt (1943*b*) has already observed that the corpus allatum (that is, the median dorsal region of the ring gland) removed from the first stage larva of *Drosophila hydei* will induce egg production when implanted into the allatectomized adult female, and has likewise suggested that the secretion responsible may perhaps be identical with the juvenile hormone reported in other groups of insects.

Twelve adult females proved by transillumination to have no ripe eggs in the ovaries* were fed, and 24 hr. later were decapitated with removal of the corpus allatum. Each was then joined to a 4th-stage nymph at 7 days after feeding, from which the forepart of the head was removed but the corpus allatum retained. Within 10 and 12 days the 4th-stage nymphs had moulted; and of the twelve adult females, five had developed ripe eggs, some only two or three, others in large numbers.

The small number of successful results is to be attributed probably to the great dilution which the juvenile hormone must suffer when shared with the adult female. In another series of experiments six adult females decapitated 24 hr. after feeding were joined to 4th-stage nymphs retaining the corpus allatum, and at the same time two corpora allata were removed from 4th-stage nymphs at 7 days after feeding and implanted into the abdomen of each female. Of the six adult females four developed large numbers of ripe eggs.

It is worth noting that in those experiments in which no egg development occurred, the characters produced by the moulting 4th-stage nymph were those of the normal 5th-stage nymph. Whereas when active development of eggs took place the characters of the nymphs showed a certain degree of differentiation towards the adult form. This result could be interpreted to mean that the developing eggs were absorbing juvenile hormone and so depriving the tissues of the moulting 4th-stage nymph of the supplies they need.

We have seen that the corpus allatum of the adult or of the late 5th-stage nymph, implanted into the abdomen of the 3rd- or 4th-stage nymph, shows evidence of secretion of juvenile hormone during the moulting of the 5th-stage nymph: in the resulting adult a small area of nymphal cuticle overlies the implant. Five adult females of this sort were decapitated at 24 hr. after feeding. All of them developed ripe eggs.

It is clear, therefore, that the corpus allatum of the adult secretes both yolk-forming hormone and juvenile hormone, and that of the 4th-stage nymph both juvenile hormone and yolk-forming hormone; while in the 5th-stage nymph neither hormone is produced. Isolation and chemical characterization of the substances in question will be necessary to prove the matter, but it appears probable, as Pfeiffer and Vogt have suggested, that the juvenile hormone and the yolk-forming hormone are one and the same substance.

DETERMINATION OF THE SECRETORY FUNCTION OF THE CORPUS ALLATUM

The experiments described earlier in this paper show, among other things, that the corpus allatum of the adult, particularly the very young adult, when implanted into the abdomen of another insect, may vary in its activities. It may secrete juvenile hormone; it may inactivate juvenile hormone; and either of these functions may predominate.

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* As described before (Wigglesworth, 1936, 1943) the ripe eggs contain parahaematin, the red colour of which is readily visible if the abdomen of the insect is examined against a bright light.

That raises the question of the control and determination of the normal function of the corpus allatum. It may be that the normal secretory function is ensured only when the integrity of the central nervous system—corpus allatum complex is unbroken, and that the timing of the activities of the corpus allatum is controlled by the brain. A few observations that bear upon this important question will be reported here.

It was suggested in a previous paper (Wigglesworth, 1936) that the characters in the early nymphal stages of *Rhodnius* are determined by the level of secretion of the juvenile hormone (inhibitory hormone). It was shown that a 4th-stage nymph deprived of its corpus allatum and caused to moult by joining it to a 3rd-stage nymph which retains its corpus allatum, again develops the characters of the 4th stage. It has now been found that if the corpus allatum of a 3rd-stage nymph is implanted into the intact 4th-stage nymph, this develops into a normal 5th stage. In other words, the corpus allatum of the host appears to control the level of concentration of juvenile hormone.

Other evidence is derived from the action exerted by implanted corpora allata at subsequent moults. When the corpus allatum of the 5th-stage nymph is implanted into the 4th-stage nymph it usually causes no visible effect (p. 4); the corpus allatum of the 4th-stage host determines the level of concentration of the juvenile hormone. Occasional slightly adultoid 5th-stage nymphs are produced. When the 5th-stage nymphs moult again they usually turn into normal adults. But, occasionally, adults are produced which are nymphoid to a varying degree. Pl. 1, fig. 11, for example, shows a nymphoid adult with short wings, intermediate genitalia, ocelli, thorax, etc. The abdominal cuticle is mostly of adult type and pigmentation but it has evenly distributed bristles as in the nymph.

It is not possible to decide in this experiment whether it is that the implanted 5th-stage corpus allatum has now become adult and is secreting the juvenile hormone again for that reason; or whether, as the result of implantation into a 4th-stage nymph, it has recovered nymphal characters and continued to secrete juvenile hormone for that reason. But in any case it is clear that the implanted corpus allatum is out of step with that of the host.

Sixth-stage nymphs can be produced by the implantation of the corpus allatum either from a young nymph (1st–4th stage) or from a mature adult. When the 6th-stage nymphs moult again their characters are variable and bear no strict relation to the stage from which the implanted corpus allatum was derived (Wigglesworth, 1936). Pl. 1, fig. 12, shows a 6th-stage nymph which was produced by the implantation of the corpus allatum from a 2nd-stage nymph into a 4th stage. When this insect moulted again it developed wholly adult characters (Pl. 1, fig. 13)—although the implanted corpus allatum had passed through only four nymphal stages. The corpus allatum of the host appears to have overcome the activity of the implant.

On the other hand, in one series of experiments in which the corpus allatum of the 3rd-stage nymph was implanted into the 4th-stage nymph, a 6th-stage nymph was produced which gave rise to a 7th stage which was entirely nymphal and showed no further differentiation towards the adult form—although the implanted corpus allatum had by that time passed through five nymphal stages and the gland in the 5th-stage nymph does not normally secrete juvenile hormone.

From these examples it is clear that the functional activity of the corpus allatum is not determined wholly by humoral means; nor is this determination wholly autonymous: it does not, for instance, produce juvenile hormone for a definite number of moulting stages. In other words, it is not the corpus allatum itself which 'counts the instars'. The mechanism responsible for this presumably resides in the central nervous system or in some part of the stomato-gastric nervous system. From the standpoint of experimental method it is obvious that the transplantation of the corpus allatum alone may give a misleading impression of its function; it is well to combine such experiments with the transfusion of haemolymph from intact insects (so-called 'parabiosis').

DISCUSSION

It was suggested in the introduction to this paper that there might be two factors involved in the control of metamorphosis in insects: (i) the corpus allatum ceases to secrete the juvenile hormone and thus permits the latent imaginal characters to be realized. (ii) Some part of the endocrine system may actively promote metamorphosis and the differentiation of the characters of the adult. The former factor has been demonstrated in Hemiptera, Orthoptera, Lepidoptera and Coleoptera, and there is some evidence for its existence in *Drosophila* (Vogt, 1943*a*, 1946). The latter factor has been regarded as characteristic of the Diptera.

In the present paper it has been shown that in *Rhodnius* the corpus allatum of the mature 5th-stage nymph not only ceases to secrete the juvenile hormone but also plays an active part in the promotion of metamorphosis. The results, however, are interpreted as indicating an active elimination by the corpus allatum of those quantities of juvenile hormone still persisting in the blood of the 5th-stage nymph.

If the corpus allatum in *Dixippus* is removed from the 3rd stage, the insect undergoes two moults and then gives rise to a diminutive egg-laying female in the 5th stage (instead of the 7th stage) (Pflugfelder, 1937). And in the cockroach *Leucophaea*, extirpation of the corpus allatum in the young nymph is followed by the appearance of a 'preadultoid' instar before the final diminutive 'adultoid' is produced (Scharrer, 1946). This presumably means that a certain amount of juvenile hormone remains in the blood from one stage to the next. There must normally be some provision for the elimination of this hormone.

It is possible, therefore, that the active removal of juvenile hormone by the corpus allatum is a general phenomenon in the metamorphosis of insects. What appears to be a 'metamorphosis promoting hormone' in the Diptera (Hadorn, 1941) may in fact be nothing more than 'inactivation of juvenile hormone'.

If we suppose that these two factors, cessation of secretion of juvenile hormone and active elimination of this hormone, differ in their relative importance in different insects, we may perhaps have the basis for a theory of metamorphosis applicable to all groups of insects.

The cessation of secretion of the juvenile hormone by the corpus allatum of *Rhodnius*, which is the chief factor that causes metamorphosis in the moulting 5th-stage nymph, is a temporary phase only. Once metamorphosis has taken place the corpus allatum begins once more to secrete the juvenile hormone. Since the juvenile hormone from the young nymphal stages will induce egg formation in the adult female and the secretion from the adult female will inhibit metamorphosis in the nymph, it seems highly probable that in *Rhodnius*, as was suggested by Pfeiffer (1945*a*) for *Melanoplus*, the juvenile hormone and the yolk forming hormone are identical.

If that is so, the hormone has quite different functions in the two stages. In the young moulting nymph it favours the intracellular system in the tissue cells which lays down nymphal structures (Wigglesworth, 1940). In the adult female it is necessary for the normal function of the follicular cells and the continued life and nourishment of the oocyte after the separation of the nutritive cords (Wigglesworth, 1936). Associated with the development of eggs in the adult female there is a much more rapid digestion of the intestinal contents. Whether this is a direct effect of the hormone on digestion and metabolism (Day (1943) and Pfeiffer (1945*b*) regard the 'gonadotropic hormone' as a 'metabolic hormone') or whether it is an indirect effect consequent upon the demands of the developing ovaries, has not been determined.* In the male it is necessary for the full activity of the accessory glands.

This dual function of what appears to be a single hormone recalls the multiple actions of thyroxin, which on the one hand has a general effect on metabolism, and on the other, has specific effects such as the induction of metamorphosis in the Amphibia.

SUMMARY

Two factors are involved in the control of metamorphosis in *Rhodnius*: (*a*) The corpus allatum of the 5th-stage nymph no longer secretes the juvenile hormone (inhibitory hormone). The latent imaginal characters can thus be realized. (*b*) The corpus allatum in the 5th-stage nymph, particularly in the later stages of moulting, actively favours the production of imaginal characters.

The evidence suggests that this activity (*b*) consists, not in the secretion of a metamorphosis promoting hormone favouring adult differentiation in the tissues directly, but in the elimination of the small quantities of juvenile hormone persisting in the blood and tissues.

If we suppose that these two factors differ in relative importance in different insects we may perhaps have the basis for a theory of metamorphosis applicable to all groups of insects.

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* It may be worth putting on record that if the ovaries of the adult female *Rhodnius* are removed there is a hypertrophy of the corpus allatum—as was first shown by Thomsen (1942) in *Calliphora* and confirmed by Day (1943) in *Lucilia* although not in *Sarcophaga*. The normal corpus allatum of a fed *Rhodnius* adult measured 71 μ; in an adult from which the ovaries had been removed 101 μ.

The corpus allatum of the mature adult *Rhodnius* again begins to secrete the juvenile hormone; and the juvenile hormone of the 4th-stage nymph will induce egg development in the adult female. It is probable that the yolk-forming hormone and the juvenile hormone are identical.

The sequence in the secretory activities of the corpus allatum, and so the number of instars, is controlled by some other centre, perhaps in the central nervous system.

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EXPLANATION OF PLATE 1

Fig. 1. Unexpanded wing developed by 5th-stage nymph which retained its corpus allatum.

Fig. 2. Unexpanded wing developed by 5th-stage nymph deprived of its corpus allatum.

Fig. 3. Normal 5th-stage nymph.

Fig. 4. Normal adult.

Fig. 5. Small patch of nymphal cuticle on the abdomen of an adult *Rhodnius*, overlying the implanted corpus allatum.

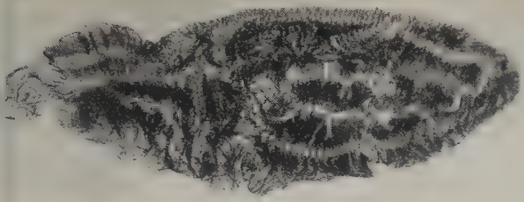
Figs. 6–9. 5th-stage nymphs showing 'adultoid' characters in varying degrees, following the implantation of corpora allata from late 5th-stage nymphs or young adults into 3rd- or 4th-stage nymphs (fuller explanation in text).

Fig. 10. 'Nymphoid' adult (explanation in text).

Fig. 11. 'Nymphoid' adult (explanation in text).

Fig. 12. 6th-stage nymph.

Fig. 13. 7th-stage adult; the same insect as fig. 12.



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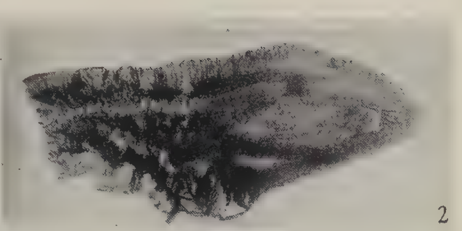

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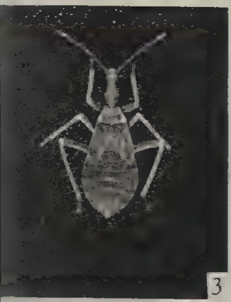

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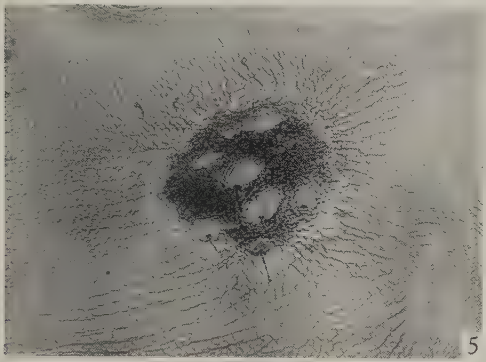

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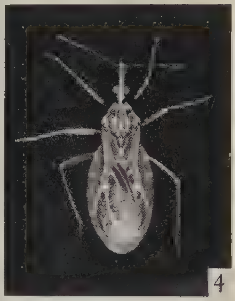

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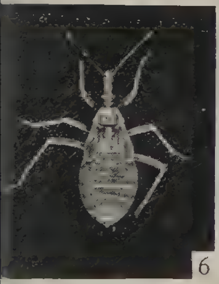

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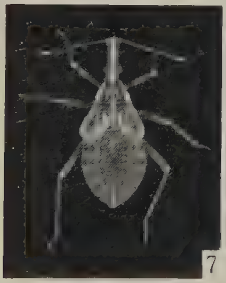

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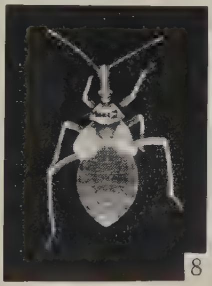

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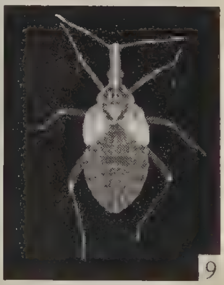

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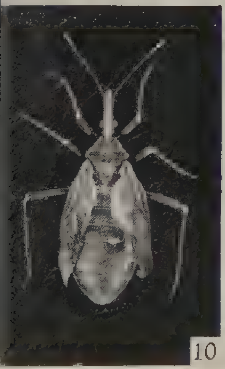

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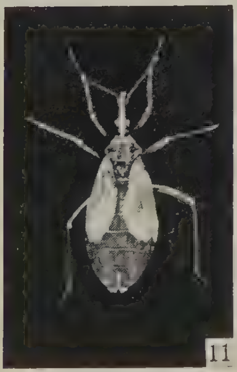

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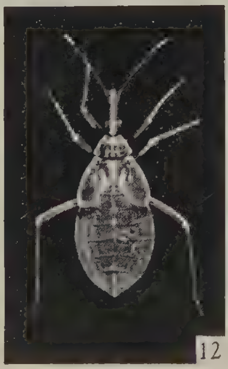

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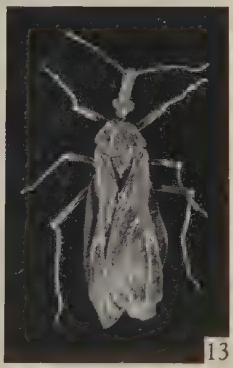

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WIGGLESWORTH—THE FUNCTIONS OF THE CORPUS ALLATUM IN *RHODNIUS PROLIXUS* (HEMIPTERA)

THE PHYSIOLOGY OF *ECHINUS ESCULENTUS* SPERMATOZOA

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(*Received* 20 *July* 1947)

(With Four Text-figures)

INTRODUCTION

Ripe sea-urchin spermatozoa are motionless in undiluted semen and intensely active when shed or artificially diluted. This activity is of short duration, presumably because the amount of energy-producing material within the spermatozoon is small, and because in pure sea water all of the endogenous substrate may not be available for combustion (Gray, 1928*a*, *b*, 1931). If semen is diluted with sea water which has been in contact with ripe eggs of the same species, the rate of oxygen consumption, the total O_2 consumed, and the mechanical activity of the spermatozoa increase (Gray, 1928*c*), though in certain conditions this effect is not observed (Carter, 1931; Hayashi, 1946). The substance responsible for the activating effect of egg water, as it is called, is said by Hartmann, Schartau, Kuhn & Wallenfels (1939) to be a substituted naphthoquinone, echinochrome, though this claim has been contested by Tyler (1939) and Cornman (1940, 1941). Carter (1932) has shown that the sperm-activating effects of egg water can be reproduced by thyroxine and certain allied substances, while Clowes & Bachman (1921) showed that propyl, allyl and cinnamyl alcohol, and related compounds also activate spermatozoa. Hartmann and Schartau (1939) found that a mixture of Naphthopurpurin and Juglone partly reproduced the effects of egg-water, both as regards activation and agglutination.

There is little information about the nature of the material within the sea-urchin spermatozoon which provides the energy for motion, the class of enzyme system or systems which contributes to the production of this energy,* or the respiratory quotient during sperm metabolism. Such information would be of intrinsic interest and also might shed some light on the fertilization reaction, by indicating what, apart from paternal hereditary material, a spermatozoon brings into the egg at fertilization.

The position is not the same in the case of mammalian semen. In mammals fertilization is internal; the spermatozoon must continue to be viable for hours, if not days, to achieve fertilization, and it is therefore not surprising that the energy for motion is normally provided outside the spermatozoon, in the form of substrates in the seminal plasma. Furthermore, there is considerable information about the metabolism of mammalian spermatozoa (Mann, 1945*a*, *b*, *c*). The existence of seminal plasma as a normal environment for mammalian spermatozoa, and its content of glycolysable substrate, recently recognized as fructose by Mann (1946), may be responsible for certain differences between the behaviour of mammalian and sea-urchin spermatozoa. On the other hand, mammalian spermatozoa can survive and move in the absence of external substrates, under aerobic conditions, for considerable periods of time.

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* Ball & Meyerhof (1940) detected spectroscopically the bands of cytochrome in the spermatozoa of *Arbacia punctulata*, and Mann (1945*b*) identified cytochromes *a*, *b*, *c* and COa_3 in ram and bull spermatozoa.

To throw some light on some of these metabolic problems, the O_2 consumption of suspensions of *Echinus esculentus* spermatozoa was investigated manometrically, in the presence of CO. The superficially more simple experiment of measuring the respiration in the presence of cyanide was not attempted because of certain difficulties encountered in the measurement of O_2 consumption in the presence of cyanide, if the evolved CO_2 is absorbed by KOH (Robbie, 1946). There are other difficulties, discussed later, when the respiration is measured by the indirect method of Warburg (1925).

Respiration through the cytochrome system is inhibited by CO in the dark, the inhibition depending upon the ratio of CO to O_2 and not upon the partial pressure of CO alone (Warburg, 1926). CO forms a compound with ferrocytochrome a_3, which is spectroscopically identifiable (Keilin & Hartree, 1939). If, therefore, the respiration of spermatozoa is photo-reversibly inhibited by CO, it follows that their metabolism is mediated through the cytochrome system.

MATERIAL

Ripe *E. esculentus* testes were dried with filter-paper and squeezed through fine-mesh bolting silk. 0·5 ml. of this viscous semen was mixed with 20 ml. of sea water or egg water. This sperm suspension was tested for activity and fertilizing power before use.

METHOD

The O_2 consumption of the spermatozoa was measured in Warburg manometers fitted with conical vessels of about 13–15 ml. capacity. The vessels contained 3·0 ml. of sperm suspension and 0·3 ml. 10% KOH in the inner cup. The latter also contained a roll of filter-paper (Whatmann no. 40) protruding 0·5 cm. over the edge of the cup and 'flowered' to increase the absorbent surface.

Experiments were done at 15° C. The shaking rate was 96–100 cycles min.^{-1} The stroke was 3·5 cm.

The gas space contained air, 90% N_2 and 10% O_2, or 90% CO and 10% O_2.

Suspensions were kept in the dark by means of lined black bags tied over the manometer vessels round the capillary tubing above the manometer stoppers. For illumination of the suspensions the bags were removed and a 200 or 500 W. lamp was switched on, some distance away from and above the water-bath. The light did not cause any heating effect as the bath was effectively cooled.

The number of spermatozoa in the suspensions was estimated by diluting a known amount of the original suspension with sea water and then doing haemocytometer counts on samples of this latter suspension, after the spermatozoa had become inactive.

RESULTS

Manometric experiments. Fig. 1 shows the results of three simultaneous experiments on samples from the same sperm suspension, one being carried out with air, one with 90% N_2 and 10% O_2, and one with 90% CO and 10% O_2, in the gas phase. All three vessels were kept in the dark for 40 min. (indicated in the graph by a black line under the time axis), after which the bags were removed and the vessels illuminated. The graph shows, first, that light and darkness have no effect on the spermatozoa with air in the gas phase; secondly, that spermatozoa, while showing a slight decrease in O_2 uptake with 90% N_2 and 10% O_2 instead of air in the gas phase, are unaffected by the alternating dark and light periods; and thirdly, that in 90% CO and 10% O_2 the sperm respiration is inhibited by about 60%. This inhibition is completely reversed by light, the slopes of the O_2 uptake curves in the N_2-O_2 mixture and the illuminated CO-O_2 mixture being parallel.

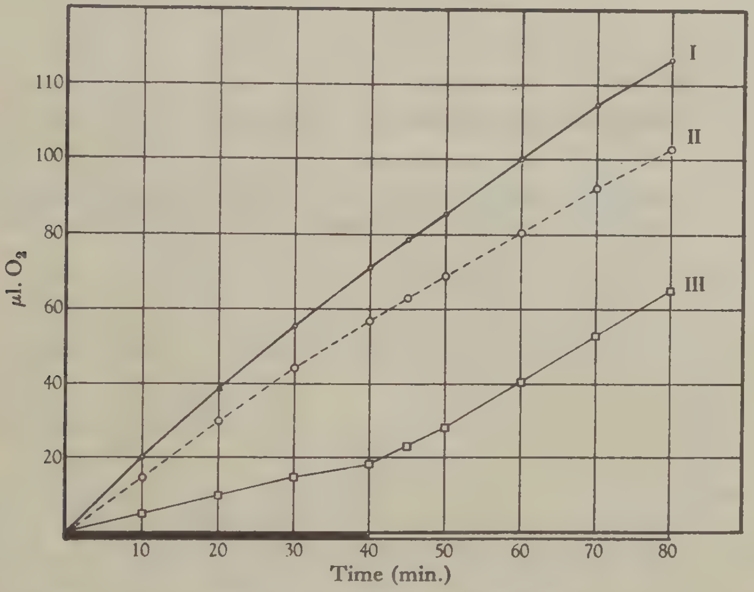

Fig. 1. Respiration of three identical *E. esculentus* sperm suspensions. I, in air; II, in a mixture of 90% N_2 and 10% O_2; III, in a mixture of 90% CO and 10% O_2. All suspensions were kept in the dark for the first 40 min. and then illuminated with a 200 W. lamp. $T°$ C., 15·1. 19 June 1947.

Fig. 2 shows the course of respiration in two identical sperm suspensions, one in air and the other in 90% CO and 10% O_2, illumination in this case being effected by a 500 W. lamp placed inside the water-bath. In these conditions the temperature in the water-bath rises, and this is immediately reflected in the rate of respiration of the sperm suspension in equilibrium with air. The effect of light in reversing the CO inhibition of the sperm suspension in equilibrium with the CO-O_2 mixture is therefore masked by the acceleration of metabolism resulting from the temperature increase.

Fig. 3 shows the O_2 consumption of three identical sperm suspensions, one having the N_2-O_2 mixture (II), and the other two having the CO-O_2 mixture in the gas phase (III and IV). All three vessels were kept for 40 min. in the dark. The O_2

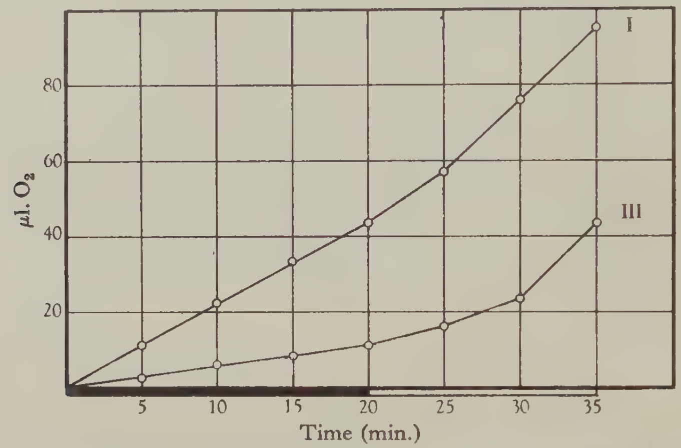

Fig. 2. Effect of temperature increase on respiration of *E. esculentus* sperm suspensions. I, in air; III, in 90% CO and 10% O_2. Illumination at $t=20$ by 500 W. lamp. $T°$ C., 15–17·5. 18 June 1947.

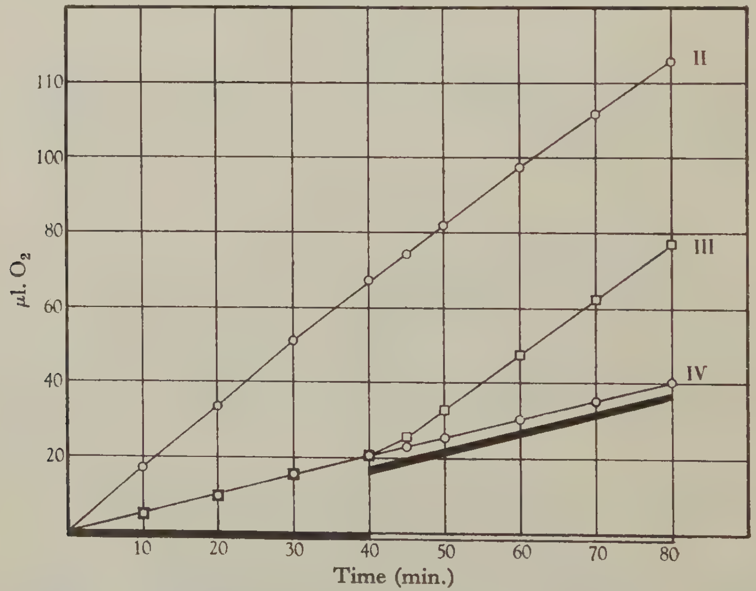

Fig. 3. Respiration of *E. esculentus* sperm suspensions. II, in 90% N_2 and 10% O_2, in darkness until $t=40$ and then illuminated; III, in 90% CO and 10% O_2, in darkness until $t=40$ and then illuminated; IV, in 90% CO and 10% O_2, in darkness throughout the experiment. Illumination by 200 W. lamp. $T°$ C., 15·1. $8{\cdot}8 \times 10^8$ sperm/ml. suspension. 20 June 1947.

consumption rates in the two suspensions with CO in the gas phase are practically identical for this period and again show about 60% inhibition. At $t=40$ min., vessels II and IV were illuminated, III being kept in the dark. While the light had no effect on the respiration of the spermatozoa in the N_2-O_2 gas mixture (II), it raised the O_2 uptake of the spermatozoa in the CO-O_2 mixture (III) to approximately the same rate as the control, showing that the CO inhibition is completely reversible. The O_2 consumption rate in the vessel left in the dark (IV) remained constant.

Fig. 4 shows the effect of alternating 30 min. periods of dark and light on a sperm suspension with 90% CO and 10% O_2 in the gas phase. The average O_2 consumption per hour for each period is plotted for purposes of comparison. There would seem to be no reason why this procedure should not be repeated for as long as the spermatozoa remain viable.

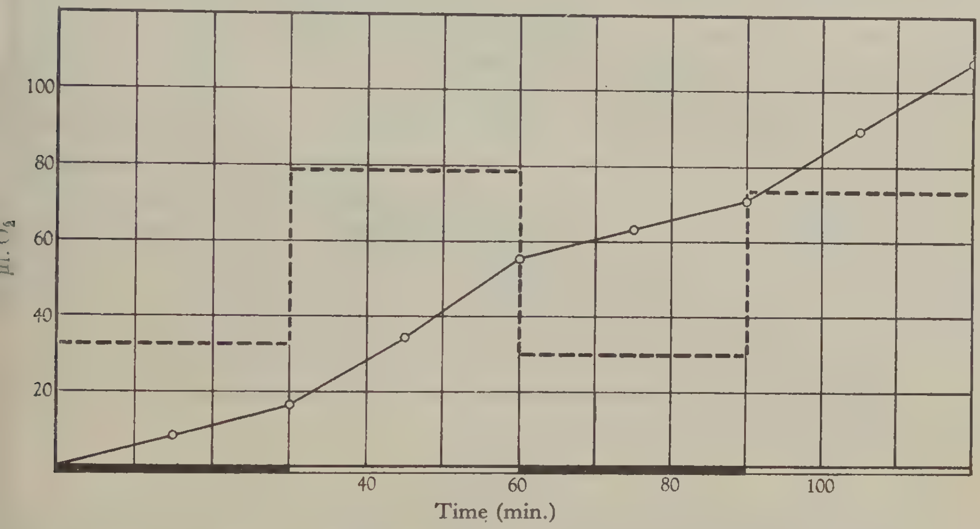

Fig. 4. Effect of alternate illumination and darkness on respiration of *E. esculentus* sperm suspension in 90% CO and 10% O_2. 0–30 min., dark; 30–60 min., light; 60–90 min., dark; 90–120 min., light. Illumination by 700 W. lamp. *T*° C., 15·1. $4{\cdot}4 \times 10^8$ sperm/ml. suspension. The broken line shows the average O_2 consumption rate per hour.

Some preliminary experiments in the presence of CO, using egg water instead of sea water as the medium for the spermatozoa, were carried out. Similar results were obtained.

Spectroscopic examination. Cytochromes *a*, a_3, *b* and *c* were clearly identified. The CO cytochrome compound was formed in the presence of CO.

DISCUSSION

The experiments described in this paper establish that the respiration (and therefore perhaps the motility) of *E. esculentus* spermatozoa are dependent on metabolic processes involving the cytochrome-cytochrome oxidase system. Egg water has no effect on this part of sperm metabolism. The fact that the cytochromes are spectroscopically identifiable does not necessarily imply that the metabolism will have photo-reversible properties under the influence of CO, though the existence of this effect, demonstrated by the manometric experiments, automatically means that the cytochrome system is involved. The experiments throw no light on the nature of the endogenous substrate. For this, measurements of R.Q. are required, though their interpretation may be difficult. The 'direct' method of measuring the ratio of CO_2 produced to O_2 consumed (Dixon, 1943) would not seem to be applicable in this case, as the method necessitates the bicarbonate content of the media in the two vessels being different. It is already known (Laser & Rothschild, 1939) that the eggs of sea urchins have different O_2 consumption rates according to the bicarbonate content of sea water. Experiments on the ratio of CO_2 produced to O_2 consumed of sperm, using a modification of Warburg's indirect method, described in the above paper, suggested that this method may not be entirely suitable for measuring the R.Q. of spermatozoa. The difficulties are probably connected with the sensitivity of spermatozoa to changes in the *p*H of the medium. For measurements of sperm R.Q., a method involving one vessel containing one suspension would be preferable, but this presents some technical difficulties.

Whatever the R.Q. may be, that is, whether protein, fat, or carbohydrate is the endogenous substrate, it is possible to make a rough calculation of the amount of substrate, believed by Lardy, Hansen & Phillips (1945) to be a phospholipid in mammalian spermatozoa, required by each spermatozoon. The O_2 consumption per hour of the uninhibited sperm suspension in Fig. 4 is 80 μl. The number of sperm per ml. was about $4{\cdot}4 \times 10^8$. As there were 3 ml. of sperm suspension in the manometer vessel the O_2 consumption per hour per spermatozoon was 6×10^{-9} μl. As 1 μl. of O_2 consumed corresponds to $1{\cdot}34 \times 10^{-6}$ g. of carbohydrate completely combusted, each spermatozoon requires 8×10^{-14} g. of carbohydrate per hour to achieve the observed consumption of oxygen.

SUMMARY

1. The O_2 uptake of *Echinus esculentus* sperm suspensions is strongly inhibited by CO. In equilibrium with a mixture of 90% CO and 10% O_2, the O_2 uptake is 60% less than that of an identical suspension in equilibrium with 90% N_2 and 10% O_2.

2. This inhibition is completely reversed by light.

3. Spectroscopic examination revealed cytochromes *a*, a_3, *b* and *c*. On treatment with CO the spectrum of COa_3 was observed.

4. These results establish that the cytochrome system is the catalytic agent which controls the respiration of sea-urchin spermatozoa.

5. The O_2 uptake of sperm suspensions in equilibrium with 90% N_2 and 10% O_2 is about 10% lower than in air.

6. Certain difficulties in measuring the R.Q. of sperm are discussed.

7. The reversible effect of CO on the O_2 uptake of spermatozoa is the same whether they are in egg water or sea water.

I am much obliged to Prof. D. Keilin, F.R.S., and his staff for reading the manuscript of this paper; and to the Director and Staff of the Scottish Marine Biological Station, Millport, where the experiments were carried out.

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A FURTHER STUDY OF THE REACTIONS OF FISH TO TOXIC SOLUTIONS

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(With Nine Text-figures)

INTRODUCTION

In a previous publication (Jones, 1947*b*) the writer has reviewed the work of Shelford & Allee (1913), Wells (1913, 1915*a*, *b*) and Shelford (1917, 1918), who studied the reactions of fish to toxic solutions with the 'gradient tank'. A modified type of apparatus was described in which the fish are confined in a horizontal glass tube, half of which may be filled with flowing tap water, the other half with flowing solution; water and solution are so sharply differentiated that in their movements up and down the tube the fish are presented with a definite concentration step, not a gradient. The reactions of the fish can be checked by reversing the directions of flow. Under these conditions *Pygosteus pungitius* reacts negatively to solutions of ethyl alcohol, chloroform, formalin, mercuric chloride and zinc sulphate, but appears to have no power of recognizing a copper sulphate solution. Into this the fish swims, to be stupefied and eventually killed.

The present paper describes further experiments on the same lines. When the work was resumed it was the writer's intention to continue using *Pygosteus* as test animal, but no further supply of this material could be obtained. Fortunately an adequate supply of *Gasterosteus aculeatus* was available and work was therefore carried on with this species. Some results have also been obtained with minnows (*Phoxinus phoxinus* L.).

SULPHIDE SOLUTIONS

The most important paper dealing with the toxicity of sulphide solutions to fish is that of Longwell & Pentelow (1935), who have shown that soluble sulphides may be formed by the anaerobic decomposition of sewage. The toxicity of sodium sulphide solutions to trout was tested and it was found that the rapidity of their effect varies with the pH of the solution; from 6·8 to 6·0 the toxicity increases slightly, from 6·8 to 7·8 it varies little, from 6·8 to 9·0 it decreases considerably. Presumably this is because in neutral or acid solution a greater proportion of the toxic substance is present in the form of unionized molecules of hydrogen sulphide. It appears to be generally recognized that the unionized molecule of hydrogen sulphide penetrates living tissue more readily than sulphide ions (see, for instance, Jacques, 1936). Stroede (1933) also deals with the mode of formation of hydrogen sulphide in natural waters and states that concentrations down to 1 mg./l. are harmful to trout; 8–12 mg./l. harmful to carp and tench. Some estimations of the toxicity of this substance to goldfish were made by Ellis (1937, p. 416). The toxic action of hydrogen sulphide resembles that of hydrogen cyanide; some observations on its effect on respiration in *Gasterosteus* have been made by the writer (1947*a*). Shelford (1917, chart 3) records two experiments to test the reactions of fish (rock bass and minnow) to sulphide solutions, but both are inconclusive.

Experiments with sulphide solutions present some difficulty. Solutions of H_2S of predetermined concentration are not easy to prepare in quantity; concentrated solutions of sodium sulphide are relatively stable, but are highly alkaline and when diluted to the degree necessary are very unstable as rapid oxidation to water and sulphur takes place. This decomposition appears to be accelerated when the solution is brought to pH 7 by the addition of hydrochloric or sulphuric acid. For satisfactory results it is necessary to employ an apparatus in which a dilute, neutral solution is automatically made up as it runs into the apparatus in which the fish are placed. The arrangement adopted in the present investigation is shown in Fig. 1.

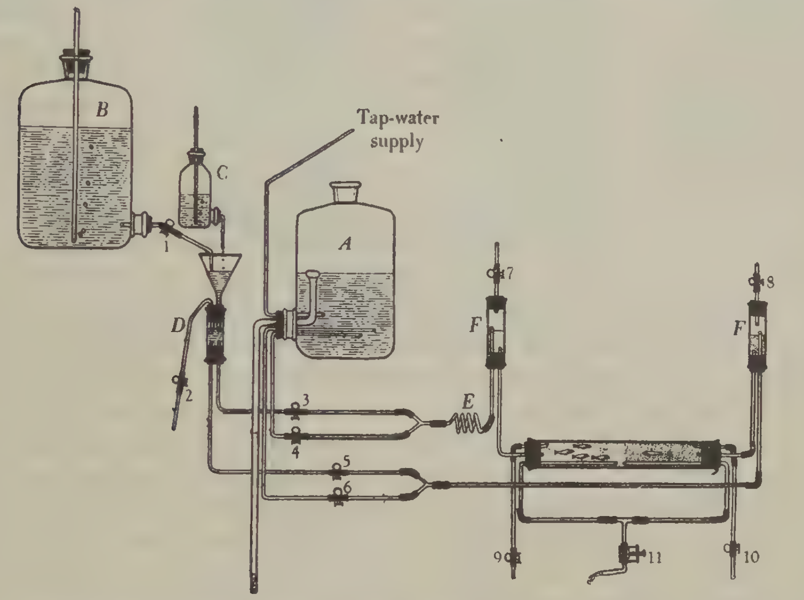


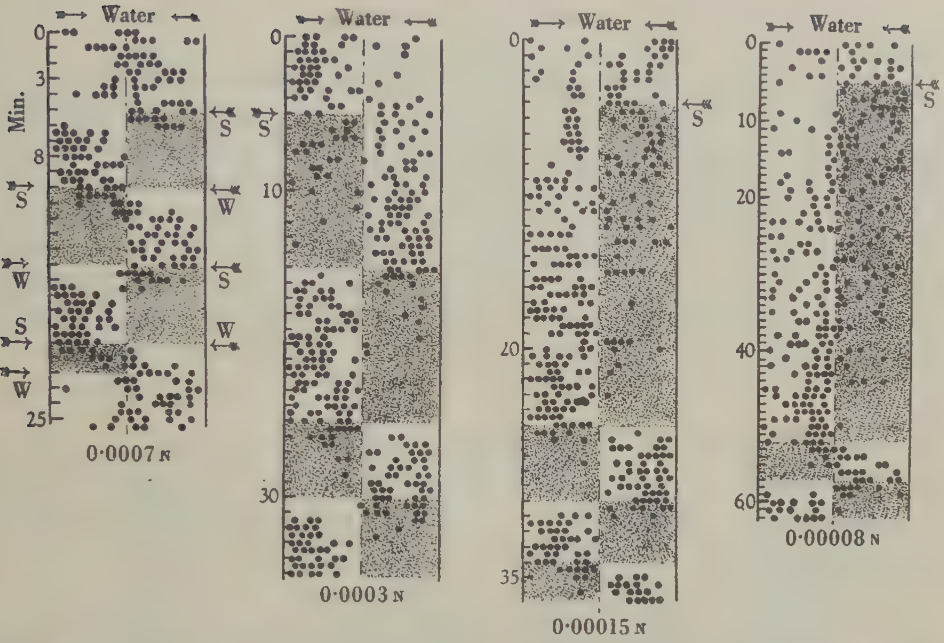
Fig. 1. Apparatus for experiments with sulphide solutions. A, 10 l. aspirator; B, 15 l. aspirator; C, 1000 ml. aspirator. 1–10, pinch clips; 11, screw clip. Bungs and rubber connexions black. Internal dimensions of tube in which fish are placed 580 × 30 mm. D, E, see text, p. 24. F, bubble traps.

Aspirator A, with inflow and outflow, provides a constant head supply of tap water. Aberystwyth tap water is fully aerated, very soft, of pH 6·8. On opening clip 4 water is delivered to the left side of the experiment tube, opening clip 6 delivers water to the right. Aspirator B contains water of the same temperature as that circulating in A; this runs into the funnel at a constant rate of 200 ml./min. Its rate of flow can be regulated exactly by adjustment of the height of the air intake. The small aspirator C contains a sodium sulphide solution of a concentration one hundred times that required for experiment. This drips into the funnel at 2 ml./min.; here again the rate of flow is regulated by adjustment of the air intake tube, and a short length of fine capillary tubing makes a satisfactory delivery tube for the dripper. In the funnel water and sulphide solution mix; sufficient sulphuric acid is added to the water in B for the mixture to have a pH of 6·8. This is most easily accomplished by starting with neutral tap water in B, withdrawing a 500 ml. sample of solution through D, titrating this with N/10 sulphuric acid and bromothymol-blue, and thus estimating the amount of acid required to be added to the supply in B when this is refilled, but a certain amount of trial and error is generally necessary before the mixture is completely satisfactory. Finally, the concentration of the mixture is checked by withdrawing another 500 ml. sample and titrating this against standard iodine and starch. The diluted, neutralized solution appears to decompose so rapidly that the concentrations measured in this way are generally slightly below values calculated from the strength of the solution in the dripper and the degree of dilution. The values given in the results which follow are based on the iodine titrations.

When not required the solution can be run to waste through D. When solution is to run into the experiment tube clip 2 is closed, opening clip 3 delivers it to the left side, clip 5 to the right. The rate of discharge from the experiment tube is regulated by screw-clip 11 so that the solution level in the mixing funnel is maintained at approximately the same height as the water supply level in A.

The part of the apparatus shown on the right resembles that used in the earlier investigation. Clips 9 and 10 are on delivery tubes which permit the expulsion of air when filling the apparatus and can also be used for drawing off samples of solution for checks of pH or concentration. The whole solution and water supply arrangement was fixed up on the experiment bench some distance from the tube in which the fish are placed, so that they are not disturbed by the operator's movements. The coil E is included to make the delivery tube to the left side equal in length to that leading to the right. The general method of experimentation and recording results followed closely that adopted in the earlier investigation. Before beginning an experiment the tap water is run for 1–2 hr. to ensure stability of temperature. The temperatures at which the experiments were conducted are recorded in the legends to the figures. It was found that provided this did not exceed 17° C. the movements of the fish are comparatively slow and deliberate. At 18–20° C., however, they tend to swim wildly up and down the tube and the results are very unsatisfactory.

A representative series of the results obtained with sodium sulphide is set out in Fig. 2. At 0·0007 N the fish display much distress immediately the solution is admitted and swim actively until all have gathered in the water zone; thereafter they avoid the solution in most definite fashion. At the lower concentrations it will be noted that the fish take a progressively longer time to react, and at 0·00008 N a definite preference for the water zone is not established until the experiment has been in progress for over 47 min. The time taken to produce this effect, that is the time for which the solution must be admitted before all of five fish definitely select



0·0007 N 0·0003 N 0·00015 N 0·00008 N

Fig. 2. The reactions of groups of five fish to 0·0007, 0·0003, 0·00015 and 0·00008 N sodium sulphide solutions. Temperature of water and solution 14° C. pH of water and solution 6·8. Fish, 28 mm. *Gasterosteus aculeatus* L.

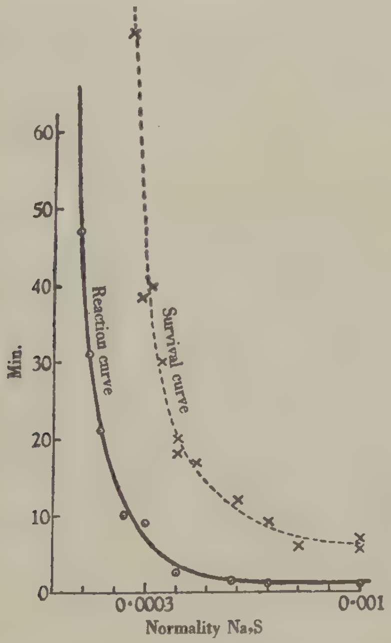


Fig. 3. 'Reaction time' curve and survival curve for *Gasterosteus* in sodium sulphide solutions. Each cross represents one survival time determination. Temp. 14° C.

the water zone and will not voluntarily enter the solution, may be termed the 'reaction time'; in Fig. 3 the reaction times observed in all the sulphide experiments are plotted.

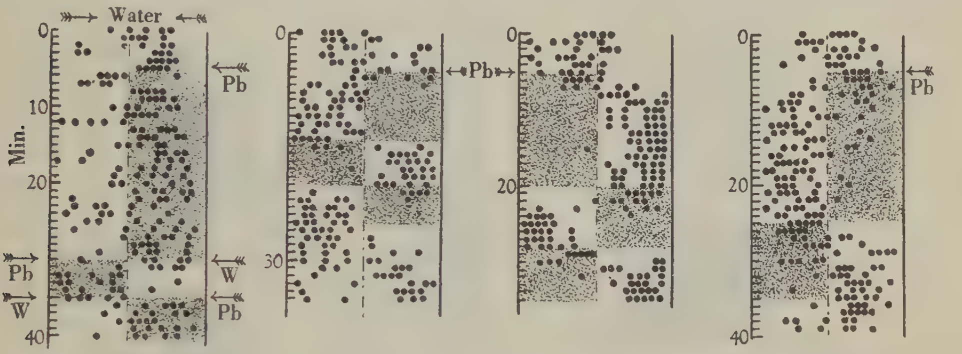
Below 0·0005 N the relation between reaction time and concentration is expressed fairly accurately by the equation $\log_{10} t = 2 - 4000c$, where t is the reaction time in minutes, c the concentration in normality. At higher concentrations theoretical values of t given by the equation are shorter than those observed, but in many of these experiments it was obvious that the 'reaction time' observed was really the time the fish took to find their way out of the solution zone, the unfavourable change in their environment being perceived in a much shorter time. Survival times for *Gasterosteus* are also given in Fig. 3, i.e. the times the fish survives when continually immersed in neutralized Na_2S solutions. It will be noted that the reaction time is substantially shorter than the survival time over the whole concentration range tested.

It is well known that the human olfactory organs can detect hydrogen sulphide at great dilution. A 1/100,000 mixture is most unpleasant and Moncrieff (1944, p. 177) states that a 10^{-7} mixture of air and H_2S is detectable. No information appears to be available as to the capability of the human organs of smell and taste for detecting the gas in solution.

Inspection of the result for 0·00015 N (Fig. 2) shows that whereas the initial reaction time is 19–21 min. the response on reversing the water-solution flow at 25 min. is much more rapid (about 4 min.), and the third response at 30 min. more prompt still. A similar result is generally observed at any other concentration at which the primary response takes some measurable period of time, as the other recordings in Fig. 2 indicate. This is not due to the fish becoming more familiar with the vessel in which they are confined, for keeping them in the apparatus for 7–8 hr. before the experiment is begun, during which period they explore their prison most thoroughly, results in no shortening of the primary reaction time. It is possible that in their many journeys up and down the tube the fish gradually learn to distinguish between water and solution, for when the reaction time is several minutes some fish may cross the water-solution junction thirty or forty times; but it seems more probable that the improved response displayed on reversing the flow results from a much more rapid development of the toxic symptoms which excite the response. Thus at the end of 25 min. (in the 0·00015 N experiment) the fish have all spent some considerable time in the solution, have developed some degree of toxaemia, and spent an insufficient time in the water zone for recovery. When the solution is now run in on the side on which they have congregated the toxic symptoms necessary to induce the negative response are developed much more rapidly, with an accompanying improvement in the speed of avoiding action. Before this hypothesis can be established a technique must be devised whereby the progress of the negative reaction is observed, together with measurement of the rate at which the sulphide solution depresses the oxygen utilization of the fish.

LEAD NITRATE

Using *Gasterosteus* as test animal the effect of lead nitrate solutions was studied over the concentration range 0·00001–0·1 N. The apparatus lay-out for these experiments resembles that adopted for sodium sulphide, but as lead nitrate is reasonably stable, even at great dilution, the dripper arrangement can be dispensed with and aspirator B filled with solution of the required concentration before beginning the experiment. With the air-intake tube in position this runs into the funnel at 200 ml./min.; this was found a better arrangement than connecting B directly with the supply tubes leading to the bubble traps, for the level of solution in the funnel provides a convenient check on the rate at which water and solution flow through the observation vessel.



0·04 N 0·004 N 0·0004 N 0·00002 N

Fig. 4. The reactions of groups of five fish (*Gasterosteus*) to 0·04, 0·004, 0·0004 and 0·00002 N, lead nitrate. Temp. 14° C. pH of solutions 5·4, 5·8, 6·4, 6·8. Survival times 3 hr., 4 hr., 5½ hr., 16 hr.

Four typical results are given in Fig. 4. At 0·1 N the experiment was not satisfactory, owing to stratification of the heavy solution, but it was evident that the fish reacted positively. At 0·04 N a better separation of water and solution was achieved; at this concentration the fish react negatively at first (note their temporary retreat at 6–7 min.), then they persist in swimming into the solution, and sooner or later are seen to be deliberately avoiding the water. When the directions of flow are reversed they select the lead nitrate zone almost immediately. Shelford (1917) noted many instances of fish reacting positively to toxic substances; this he attributed to the fish failing to recognize the solution, swimming into it and becoming 'intoxicated'.

Some efforts were made to elucidate this positive reaction, but without complete success. The pH of 0·04 N lead nitrate is 5·4; this is not an attraction for *Gasterosteus* which is fairly definitely negative to water of this degree of acidity (see Fig. 5*b*). Another explanation that might be suggested is that the fishes' chemical sense is dulled or destroyed by exposure to concentrated lead nitrate, for it is well known that washing out the mouth with a strong astringent temporarily impairs the sense of taste very considerably. Fig. 5*a* records one of the experiments carried out to test this theory. Five fish were placed in the observation vessel and their reaction to 0·0007 N lead nitrate tested in the usual way; the result, a prompt negative response. Then after 5 min. of double water flow 0·04 N lead nitrate was run in at both ends for 15 min. This was washed out with water and then 0·0007 N lead nitrate was run in on one side. Though the fish showed obvious distress after their 15 min. exposure to the more concentrated solution, they reacted to the dilute solution negatively, and quite as promptly as before.

Next the reaction of *Gasterosteus* to calcium nitrate was examined. To dilute solutions (e.g. 0·001 N) the fish appear quite indifferent, but to 0·04 N $Ca(NO_3)_2$ a positive reaction is displayed. A similar result is observed with 0·03–0·05 N sodium nitrate or 0·03–0·07 N sodium chloride. These results suggested that the osmotic pressure of the 0·04 N lead nitrate might be its attractive feature and the reaction of *Gasterosteus* to glucose solutions of similar osmotic pressure was tested. Here, however, the reaction of the fish was negative; they preferred the water and if they ventured into the glucose appeared irritated, swimming in an erratic and nervous manner. It has to be admitted that at the present stage a satisfactory explanation of the positive reaction of the stickleback to concentrated lead nitrate is not forthcoming; in a subsequent section of this paper it will be shown that *Gasterosteus* is positive to dilute ammonia solutions.

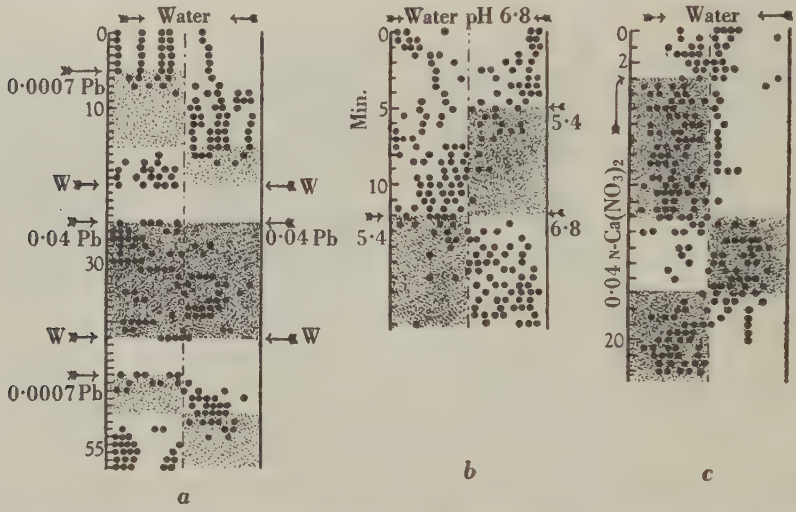


Fig. 5. *a*, the reaction of *Gasterosteus* to 0·0007 N lead nitrate before and after 15 min. exposure to 0·04 N lead nitrate. *b*, the reaction of *Gasterosteus* to water of pH 5·4. *c*, the reaction of *Gasterosteus* to 0·04 N calcium nitrate, pH 6·2.

At 0·01 N the reaction is vague, the fish keep wandering restlessly to and fro, and on further dilution (0·004 N) a negative reaction is displayed. At 0·0004 N the response is similar, and equally prompt; on running in the solution the fish are greatly agitated and quickly choose the water zone. Here they remain and should one venture to the centre of the experiment tube it pauses, gobbles, and retreats with obvious distaste. At 0·00002 N (approximately 2 mg. Pb/l, survival time about

16 hr.) a delayed, but nevertheless definite negative reaction is displayed. The movements of the fish are leisurely and in their visits to the solution zone they display little irritation. On reversing the water-solution flow (at 25 min.) they again have some difficulty in selecting the water zone and some fish swim up and down the tube ten or more times before making up their minds. One experiment was run at 0·00001 N; in this a vague negative reaction developed in about 30 min.

In Fig. 6 the reaction time-concentration curve is plotted together with some reaction times observed with zinc sulphate and with lead nitrate when the minnow (*Phoxinus phoxinus*) is used as test animal. Five fish were used at each concentration. The minnow is much more sensitive than the stickleback; 0·0007 N lead nitrate is avoided immediately, the fish retreating from the solution as it enters, and even a 0·000004 N solution (0·4 mg./l.) excites a definite negative response in 8 min. Single fish experiments in which all the movements of the fish were recorded were also made with *Phoxinus*; three results which illustrate very well the reactions of the minnow to dilute lead nitrate are given in Fig. 7; note the almost immediate rejection of the solution at 10^{-4} N, the several trials and eventual rejection of the solution at 10^{-5} N.

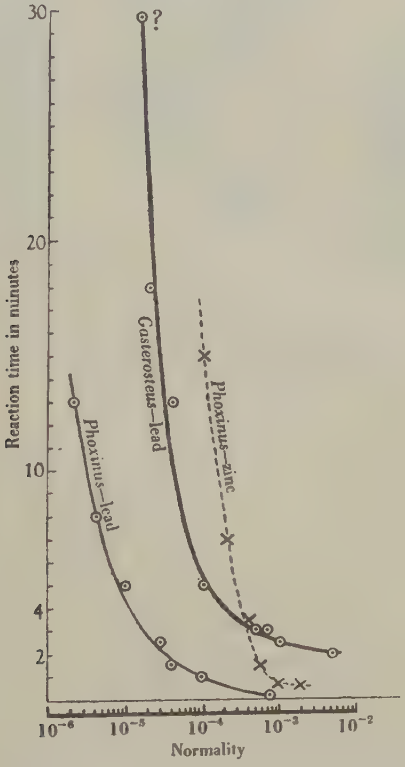
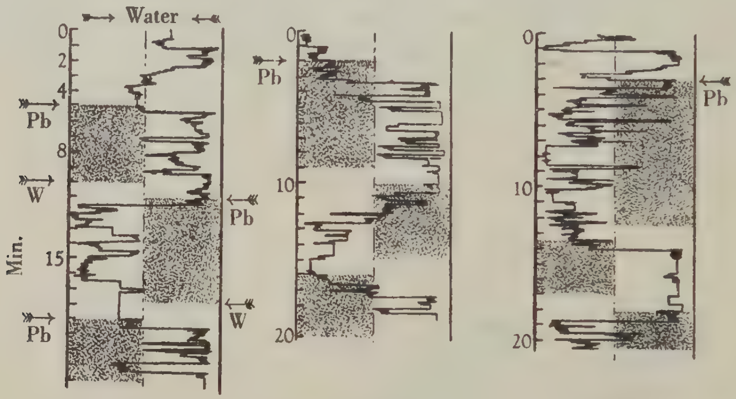


Fig. 6. Reaction times for *Phoxinus* (lead nitrate and zinc sulphate), and for *Gasterosteus* (lead nitrate). Temp. 14° C.

It is possible that the minnow can detect, but is not irritated by, lead nitrate solutions of greater dilution than 0·000004 N, but even if this represents the threshold it is remarkably low. Moncrieff (1944, p. 111) supplies some information on the sensitivity of the human sense of taste to heavy metal salts, stating that silver nitrate is just detectable in 0·003 % solution. This would be about 0·00018 N, or 19 mg./Ag/l.



0·0001 N 0·00003 N 0·00001 N

Fig. 7. The reactions of single fish (*Phoxinus*) to 0·0001, 0·00003 and 0·00001 N lead nitrate. pH of all solutions 6·8. Temp. 16° C.

WATER OF ABNORMAL HYDROGEN-ION CONCENTRATION

It is well known that the hydrogen-ion concentration of the water is one of the most important factors determining the type of fauna found in any aquatic environment and observations in field and laboratory on the pH ranges different animals can withstand are in plentiful supply. Using the goldfish as test animal Ellis (1937, p. 409) has studied the lethality of eleven acids, and (1937, p. 379) reviews the results of a number of workers which seem to show that fresh-water fish have a considerable tolerance for variations in hydrogen-ion concentration. On the other hand, little study appears to have been made of the motor reactions of fish to water of abnormal acidity or alkalinity. Wells (1915*a*) made some attempt at the problem, but his results are somewhat vague; using the gradient tank and three different species of fish he decided that fish are negative to neutrality in favour of slight acidity According to Shelford (1929, p. 480, footnote) Wells took neutrality as the 'turning point' of phenolphthalein, presumably about 9·0.

The reaction of *Gasterosteus* to acid and alkaline water has been studied over the pH range 3·2–12·0. In every experiment the fish were offered acid or alkaline water as an alternative to the Aberystwyth tap water (pH 6·8), in which they were kept until used for experiment, and which has a pH approximately that of the river from which they were obtained. The scheme of apparatus resembles that of Fig. 1. Aspirator A supplies water of pH 6·8; aspirator B is replaced by a duplicate of A, providing a second constant head supply from the same pipe. This runs at 200 ml./min. into the funnel to dilute, about one hundred times, a hydrochloric acid or sodium hydroxide solution dripping steadily from C. Samples of the mixture are withdrawn at D, their pH determined, and the concentration of the supply in C and its rate of flow adjusted until the mixture has the pH required. This is finally checked by the examination of samples drawn at 9 or 10.

Colorimetric estimation was found quite satisfactory for the acid range; for the alkaline range phenol-thymol-phthalein is satisfactory up to pH 10·4. Above this value no completely reliable indicator could be found, though alizarin S, methyl blue, nile blue and brilliant cresyl blue were tried, and pH determinations over the range 10·4–12·0 were therefore checked with a Cambridge hydrogen-ion apparatus.

One of the experiments with acid water is that recorded in Fig. 5*b*, already referred to. All the other hydrogen ion experiments with *Gasterosteus* are summarized in Fig. 8. To water of acidity greater than pH 5·4 the stickleback is definitely negative, avoiding it with the usual symptoms of discomfort. The writer (1939) has shown that the hydrogen ion threshold is about pH 5·0, which the stickleback survives for 9 days. Using nitric acid Carpenter (1927) obtained a similar result with *Phoxinus*.

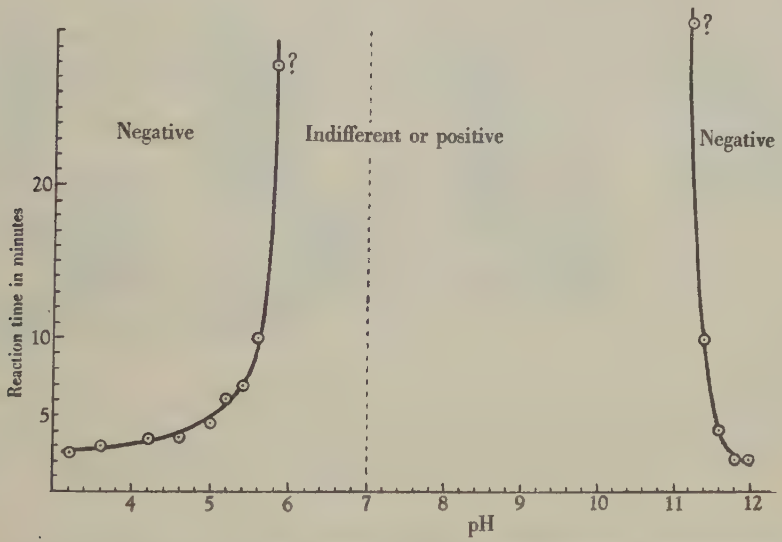


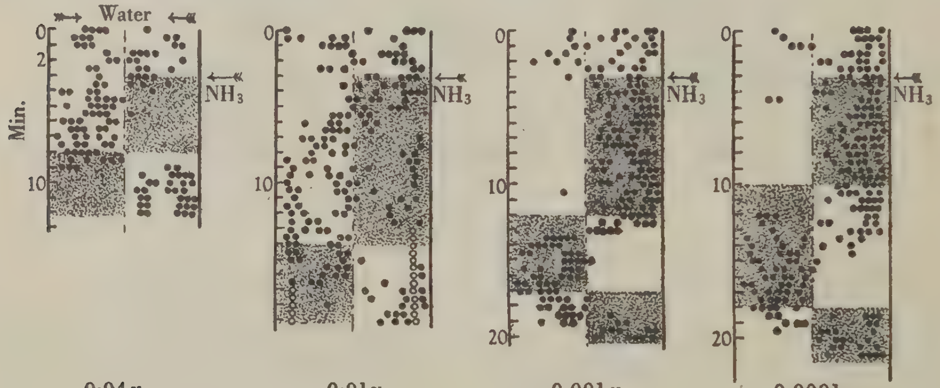
Fig. 8. The reaction of *Gasterosteus* to acid and alkaline water. Five fish used for each reaction time determination. Temp. 14° C.

At 5·8 a very vague negative reaction is displayed and from 6·0 to 7·0 the fish appear indifferent. Water of pH 7·0 to 11·0 similarly excites no definite response; if any reaction at all develops in the course of 30 min. it is an indefinite preference for the alkaline zone. At 11·4 a fairly definite negative reaction is developed, and still more alkaline water is very quickly avoided with widely distended mouth and other symptoms of distress, the alkali portion of the reaction time curve dropping much more steeply than the acid.

AMMONIA

The toxicity of ammonia solutions to fish has been studied by a number of investigators interested in pollution problems, including Ellis (1937), Shelford (1917), and Mason-Jones (1930). Grindley (1946) has measured the toxicity of ammonium chloride to rainbow trout, his experiments seem to place the threshold at 314 mg. NH_4Cl/l. (about 0·006 N). Shelford concluded that fish are unable to recognize even highly toxic solutions of the gas, but the two experiments he records (1917, chart 2) are not very satisfactory for the fish were driven to the ammonia side of the gradient tank, nor does he state the actual concentration tested.

Four of the writer's results with *Gasterosteus* are given in Fig. 9. The apparatus arrangement used for lead nitrate was found satisfactory; the ammonia solutions were prepared by diluting a normal solution of ammonia standardized with N/10 HCl, the concentrations of the solutions being checked by titrating 500 ml. samples at the end of the experiments, and the pH values given are those of samples drawn from the experiment tube.



0·04 N pH 10·6 | 0·01 N pH 10·4 | 0·001 N pH 9·8 | 0·0001 N pH 8·8

Fig. 9. The reactions of groups of five fish (*Gasterosteus*) to 0·04, 0·01, 0·001 and 0·0001 N ammonia. Survival times 2 min., 4½ min., 35 min., over 24 hr. Temp. 16° C. Open circles represent dead fish.

To 0·04 N ammonia the stickleback is promptly negative, a negative reaction is also developed towards a 0·01 N solution, but somewhat tardily, though this concentration is highly toxic (survival time 4½ min.). Two fish persisted in swimming into the solution with the result that they died suddenly with convulsive movements and widely distended mouths. With further dilution the negative response disappears and is replaced by a most definite positive reaction; in the 0·001 N experiment note how the fish avoid the water and gather in the solution zone within 2 min. of the flow reversal at 12 min. At 0·0001 N the reaction is still positive.

In dilute ammonia solutions we apparently have a clear case of positive reaction to a lethal substance. As the osmotic pressure of the solution differs little from that of the tap water, and as the pH of the solution does not appear attractive, we can only conclude like Shelford that the toxic substance 'intoxicates' the fish in some way.

Furthermore, it will be noted that the general result with ammonia solutions is the converse of that observed with lead nitrate, to which *Gasterosteus* is positive at high concentrations, negative at great dilution. Hence, when the reaction of fish to any toxic substance is studied it is important to cover a wide concentration range, otherwise any conclusions based on the results may be inaccurate.

GENERAL OBSERVATIONS

Reviewing the whole of the writer's observations on the behaviour of fish to toxic solutions the following general observations may be made. The reaction observed may be of three types: (1) Positive, the fish being attracted by the toxic substance itself (ammonia), possibly by some other property of the solution, such as its osmotic pressure (concentrated lead nitrate). (2) A complete failure to distinguish between the solution and water, with a pseudo-positive reaction when the substance in question is highly toxic (copper sulphate). (3) A negative reaction, the promptness of which depends on the concentration of the solution, and which may be maintained down to the threshold of toxicity (sulphide). Fish may be positive to a substance in high concentration, negative to the same substance in dilute solution, and vice versa.

Even when substances whose physiological effects are similar are considered the fishes' capability of detecting and rejecting them is not related to their degree of toxicity. The four heavy metal salts tested, in decreasing toxicity form the order mercuric chloride, copper sulphate, lead nitrate, zinc sulphate. According to the stickleback's ability to detect and avoid their presence the order is lead, mercury, zinc, copper.

Lastly it is evident that the sensitivity of one fish to a particular substance may differ considerably from that of another, for the minnow is some ten times as sensitive to lead nitrate as the stickleback.

SUMMARY

This paper records some further observations on the reactions of fish to toxic solutions. The method of experimentation resembles that described in a previous paper by the writer (Jones, 1947*b*). In every case the solution is presented as an alternative to the Aberystwyth tap water, which is well aerated, very soft, of pH 6·8.

In experiments with sodium sulphide a supply system is arranged in which dilute sodium sulphide solution, brought to pH 6·8 by the addition of sulphuric acid, is automatically made up as it runs into the observation vessel. *Gasterosteus aculeatus* L. reacts negatively to a 0·001 N solution almost immediately; at greater dilution the 'reaction time' lengthens, at 0·00008 N is about 47 min. Over the concentration range tested the reaction time is always shorter than the survival time.

Gasterosteus is positive to 0·04 N lead nitrate. As a positive reaction is also displayed to equivalent concentrations of calcium nitrate, sodium nitrate and sodium chloride it is possible that the osmotic pressure of the solution is its attractive feature.

At 0·01 N the positive response to lead nitrate disappears and at 0·004 N is replaced by a very definite negative reaction which is maintained down to 0·00002 N. The minnow (*Phoxinus phoxinus* L.) is also negative to dilute lead nitrate and will detect and avoid a 0·000004 N solution.

Gasterosteus will avoid water more acid than pH 5·6 or more alkaline than pH 11·4. Over the range 5·8–11·2 the fish are indifferent or very vaguely positive.

Gasterosteus is negative to 0·04 and 0·01 N ammonia solution, positive to 0·001 and 0·0001 N. The general result with ammonia is thus the converse of that observed with lead nitrate.

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THE OXYGEN REQUIREMENTS AND THERMAL RESISTANCE OF CHIRONOMID LARVAE FROM FLOWING AND FROM STILL WATERS

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(With Three Text-figures)

Extensive surveys of the distribution of chironomid larvae have established the fact that the majority of species are strikingly restricted in their habitat: the causes of this specialization, however, remain largely undetermined. For a few species comprehensive ecological work has made it possible to deduce the factors important in determining distribution (Thienemann, 1923, 1928; Lenz, 1925), but an experimental attack on the physiology and behaviour of larval and adult chironomids in relation to their choice of habitat remains almost a virgin field.

In other groups of aquatic invertebrates experimental work has indicated that related forms from different habitats may have well-marked physiological differences. Various species of ephemerid nymphs and caddis larvae from streams, for instance, have higher metabolic rates than related species from stagnant water, and the former are more sensitive to oxygen lack than the latter (Fox & Simmonds, 1933; Fox, Simmonds & Washbourn, 1935). May-fly nymphs from swift streams are less resistant to high temperatures than those from slow-moving waters (Whitney, 1939), and the heat tolerance of other aquatic organisms has been shown to be adapted to the temperature conditions of their environment (Plateau, 1872; Mason, 1939).

In order to find the extent to which the physiological requirements of chironomid larvae are related to their special habitats I have made measurements of certain physiological processes in the larvae of a number of species from different types of environment. The rate of oxygen consumption, capacity for anaerobiosis and resistance to high temperatures were measured. As far as possible closely related species with different habitats were selected for comparison.

(1) OXYGEN CONSUMPTION

The oxygen requirements of chironomid larvae of four species were studied. The larvae fell into two comparable pairs, the members of each pair being closely related and of about the same size. One member of each pair occurs in a stream and the other in a still-water environment. The following species were used: *Anatopynia varia* (Fabr.) (Tanypodinae) from a stagnant ditch; *A. nebulosa* (Mg.) from a stream. *Chironomus longistylus* Goet. (Chironominae) from a stagnant ditch; *Tanytarsus brunnipes* (Zett.) (Chironominae) from a stream. Final instar larvae of all species were used, except *Chironomus longistylus* in which case young larvae, 6–7 mm. in length, were selected in order to be more nearly comparable with the 7 mm. larvae of *Tanytarsus*. The three last-mentioned species have sufficient haemoglobin in the blood to make the larvae appear distinctly red; *Anatopynia varia*, however, contains much less, the blood being only pale pink, and in some individuals almost colourless. The larvae were usually collected on the day before the experiments and were kept overnight in running water in shallow dishes with a thin layer of mud. Larvae which had been more than 2 days in the laboratory were never used.

The oxygen consumption of the larvae was determined by the method described by Ewer (1942). Between twelve and twenty *Chironomus* or *Tanytarsus* larvae, or six *Anatopynia*, were used each time.

The results of these experiments are given in Tables 1–4 and Fig. 1. The ditch and stream species differ strikingly in their oxygen requirements. Both *Tanytarsus brunnipes** and *Anatopynia nebulosa*, from streams, have a higher metabolic rate at

Table 1. *Oxygen consumption of young* Chironomus longistylus *Goet. larvae at* 17° C.

Oxygen concentration (ml./l.)	Oxygen consumption (cu.mm./g. (wet wt.)/hr.)	
	Separate values	Means
6·01–7·00	444, 331, 417, 200, 342, 219, 140, 337, 452	320 ± 37
5·01–6·00	366, 335, 311, 280, 308, 366, 360, 394, 296, 396, 342, 323, 543, 375, 304, 202, 107	330 ± 21
4·01–5·00	292, 266, 247, 312, 259, 379, 244, 284, 381	296 ± 9
3·01–4·00	309, 252, 313, 310, 258, 244, 505, 328, 390, 316	313 ± 22
2·01–3·00	268, 236, 297, 240, 245, 356, 294, 359, 315, 287, 324, 196, 314, 225, 440, 464	304 ± 11
1·01–2·00	350, 219, 379, 215, 279, 330, 315, 267, 291, 277, 213, 377, 266, 264, 184, 332, 283, 295	285 ± 13
0·51–1·00	168, 220, 247, 245, 297, 205, 228, 213, 159	220 ± 13
0·00–0·50	45, 62, 205, 68, 185	117

Table 2. *Oxygen consumption of* Tanytarsus brunnipes (*Zett.*) *larvae at* 17° C.

Oxygen concentration (ml./l.)	Oxygen consumption (cu.mm./g. (wet wt.)/hr.)	
	Separate values	Means
6·01–7·00	364, 484, 360, 270, 434, 356, 394, 590, 666, 531, 700, 482	469 ± 38
5·01–6·00	500, 451, 486, 304, 233, 336, 313, 344, 565, 621, 466, 440, 635, 448, 301	430 ± 31
4·01–5·00	305, 418, 361, 354, 363, 210, 371, 440, 435, 467, 238, 399, 315, 320, 404, 296, 467, 436	367 ± 9
3·01–4·00	318, 286, 287, 341, 331, 219, 232, 315, 378	301 ± 17
2·01–3·00	297, 366, 280, 327, 218, 310, 422, 390, 381, 368, 303, 268, 307, 294, 350, 443, 426	342 ± 8
1·01–2·00	250, 119, 329, 248, 251, 315, 237, 263, 226, 224	246 ± 16
0·50–1·00	47, 146, 128, 176, 70, 198, 201, 162	141 ± 20

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* Other data of the oxygen consumption at various oxygen pressures of *T. brunnipes* have already been published (Walshe, 1947). The experiments giving these data were made on larvae from a different habitat and at a different season: these factors must account for the slight differences between the two sets of data.

Table 3. *Oxygen consumption of* Anatopynia varia (*Fabr.*) *larvae at* 17° C.

Oxygen concentration (ml./l.)	Oxygen consumption (cu.mm./g. (wet wt.)/hr.)	
	Separate values	Means
6·01–7·00	348, 156, 300, 428, 503, 503, 428, 287, 214, 207	337 ± 40
5·01–6·00	402, 304, 263, 324, 326, 430, 511, 271, 413	360 ± 28
4·01–5·00	96, 357, 115, 256, 307, 353, 273, 356, 340, 276, 198, 224, 275, 242	262 ± 22
3·01–4·00	218, 194, 231, 394, 247, 199, 167, 426, 386, 296, 413	288 ± 30
2·01–3·00	340, 357, 350, 414, 195, 247, 200, 381, 429, 441	335 ± 29
1·01–2·00	67, 216, 132, 251, 339, 244, 314, 167, 134, 255, 268, 210, 286, 221, 182	220 ± 20
0·00–1·00	75, 1, 38, 120, 195, 179, 92, 33, 46, 225, 88, 335, 352, 457	160 ± 36

Table 4. *Oxygen consumption of* Anatopynia nebulosa (*Mg.*) *larvae at* 17° C.

Oxygen concentration (ml./l.)	Oxygen consumption (cu.mm./g. (wet wt.)/hr.)	
	Separate values	Means
6·01–7·00	519, 253, 542, 371, 423, 346, 440, 370, 324, 454, 466, 612, 668, 223	429 ± 34
5·01–6·00	510, 183, 474, 264, 395, 209, 231, 206, 258, 277, 337, 474, 388	324 ± 33
4·01–5·00	194, 228, 232, 268, 306, 305, 412, 235, 288, 324, 197, 213, 279, 301, 221, 224, 479, 300, 315, 170	275 ± 16
3·01–4·00	134, 267, 299, 174, 157, 232, 293, 261, 215, 290, 232, 129, 197, 250, 173, 118, 453, 611, 131	243 ± 27
2·01–3·00	179, 169, 67, 228, 362, 208, 247, 275, 134, 196, 140, 162, 208, 189	197 ± 19
1·01–2·00	85, 67, 176, 20, 125, 24, 138, 152, 104	99 ± 18
0·00–1·00	86, 39, 132, 98, 106	92

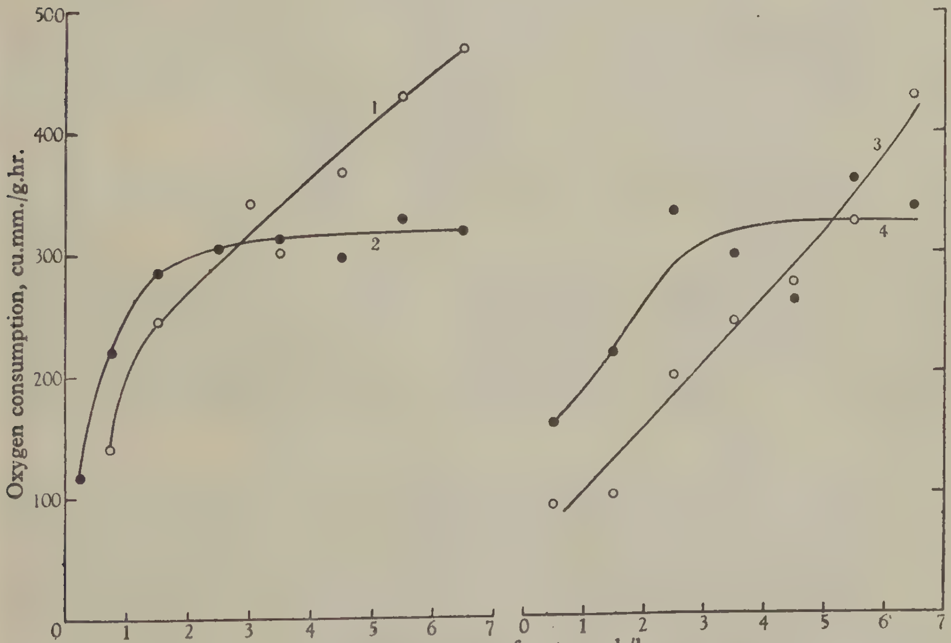

Fig. 1. Rates of oxygen consumption at 17° C. of chironomid larvae at various concentrations of dissolved oxygen. (1) *Tanytarsus brunnipes* (stream); (2) *Chironomus longistylus* (ditch); (3) *Anatopynia nebulosa* (stream); (4) *A. varia* (ditch). Data from Tables 1–4.

air saturation of the water than the corresponding ditch species; but at lower oxygen contents the oxygen consumption of the stream species falls rapidly, the relation between oxygen consumption and oxygen content of the water being roughly linear. The rate of oxygen consumption of the two stagnant water species, however, is almost independent of the oxygen content until this falls to the value of 3 ml./l. (44% air saturation).

The rates of heart beat, in fully aerated water at 17° C., of the four species were found to be 32·4 ± 1·1 beats per min. in *Anatopynia varia* as compared with 32·4 ± 1·0 in *A. nebulosa*, and 72·9 ± 5·1 in *Chironomus longistylus* compared with 64·4 ± 2·1 in *Tanytarsus brunnipes*. There is thus no significant difference in the rate of blood circulation in the comparable ditch and stream species in spite of the differences in oxygen consumption. This is unlike the case of the ephemerid nymphs *Cloeon* and *Baetis* (Fox & Simmonds, 1933).

(2) SURVIVAL UNDER ANAEROBIC CONDITIONS

A comparative study was made of the capacity of larvae from different habitats to live under anaerobic conditions. A list of the species used and the habitats from which they were obtained is given in Table 5. In all cases final instar larvae were used. The length of survival without oxygen was measured by enclosing the larvae in bottles of nitrogen-saturated water, which were then kept in the dark at room temperature and the numbers of dead noted at intervals. In all cases larvae were enclosed in the proportion of 0·2 g. to 400 ml. water: the number of animals used varied from 5 to 25 per bottle according to the size of the larvae. Five bottles of each species were used.

Table 5. *Survival times of various chironomid larvae under anaerobic conditions*

No. on Fig. 2	Species	Subfamily	Habitat	50% alive (hr.)
1	*Tanytarsus brunnipes* (Zett.)	Chironominae	Stream	8
2	*Prodiamesa olivacea* (Mg.)	Diamesinae	Stream	10
3	*Procladius choreus* (Mg.)	Tanypodinae	Stream	12
4	*Anatopynia nebulosa* (Mg.)	Tanypodinae	Stream	14
5	*Chironomus albimanus* Mg.	Chironominae	Stream	16
6	*C. rubeculosus* Mg.	Chironominae	River	20
7	*Anatopynia varia* (Fabr.)	Tanypodinae	Ditch	46
8	*Tanypus punctipennis* Mg.	Tanypodinae	Stagnant backwater	50
9	*Chironomus longistylus* Goet.	Chironominae	Ditch	68
10	*C. paganus* Mg.	Chironominae	Concrete trough	101

The results of these experiments are given in Fig. 2, which shows clearly that the species fall into two well-defined groups: one in which the capacity to live anaerobically is small, 50% of the larvae being dead after 8–20 hr., and the other in which the animals remain alive much longer, the 50% death points ranging from 46 to 101 hr. The distinction between the two groups is unrelated to any systematic or morphological differences in the larvae, for the various species of *Anatopynia* and *Chironomus* are seen to have very different susceptibilities. On the other hand, the distinction between the two groups can clearly be correlated with differences in habitat. The group with a slight capacity for anaerobiosis is composed of species taken from running waters, while the larvae in which the capacity is greater are all from stagnant water environments. This is comparable with the case of the ephemerid nymphs and caddis larvae of Fox *et al.* (1933, 1935).

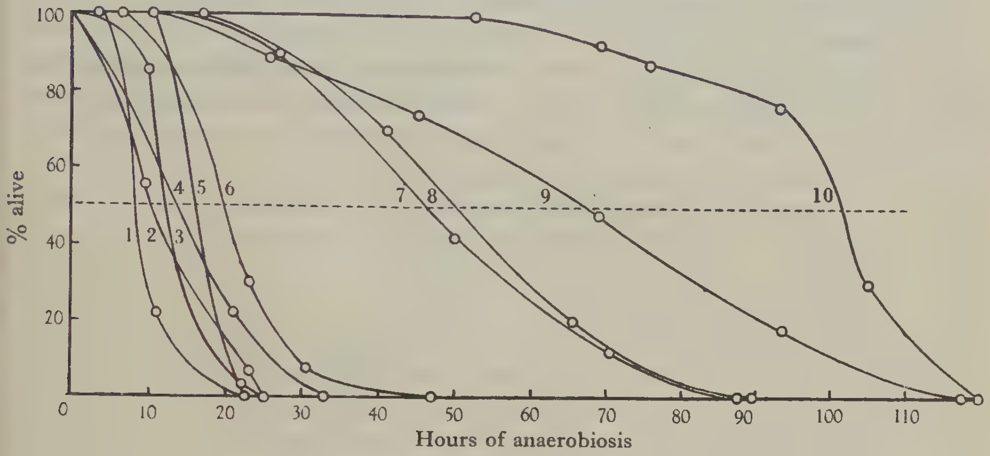

Fig. 2. Duration of life under anaerobic conditions of various chironomid larvae. Species used (nos. 1–10) given in Table 5.

(3) THERMAL RESISTANCE

Experiments were made to determine the heat tolerance of larvae from different habitats. Table 6 gives a list of the seven species with their habitats; fully grown larvae were used. The thermal resistance was determined by placing twenty larvae in an open glass vessel containing 100 ml. of air-saturated water, and keeping them in a thermostat at an appropriate temperature for 22 hr. The number which died during that time was then counted. Temperatures between 23 and 39° C. were used. The water was not aerated during the experiments; consequently less oxygen was available at the higher temperatures. Even at the highest temperatures, however, the diminution of dissolved oxygen could not alone have been responsible for the death of the larvae, but it possibly modified their capacity to resist high

Table 6. *Heat resistance of various chironomid larvae*

No. on Fig. 3	Species	Subfamily	Habitat	Thermal index (° C.)
1	*Tanytarsus brunnipes* (Zett.)	Chironominae	Stream	29
2	*Prodiamesa olivacea* (Mg.)	Diamesinae	Stream	30
3	*Anatopynia nebulosa* (Mg.)	Tanypodinae	Stream	30·5
4	*Chironomus riparius* Mg.	Chironominae	Concrete trough	34·5
5	*C. albimanus* Mg.	Chironominae	Stream	35
6	*C. longistylus* Goet.	Chironominae	Ditch	35·5
7	*Anatopynia varia* (Fabr.)	Tanypodinae	Ditch	38·8

temperatures.* In nature, however, high temperatures would almost always be accompanied by diminished oxygen concentrations: the larvae would thus be influenced by a combination of the two factors.

Fig. 3 shows the decline in numbers of larvae remaining alive after 22 hr. at various temperatures. The capacity to withstand high temperatures varies: taking as a thermal index the temperature at which 50% of the larvae are dead in 22 hr., the values range from 29 to 39° C. The species from flowing water had thermal indices of 29, 30, 30·5 and 35° C., those from stagnant water had indices of 34·5, 35·5 and 38·8° C. Of special interest is the remarkable power of thermal resistance of *Anatopynia varia*.

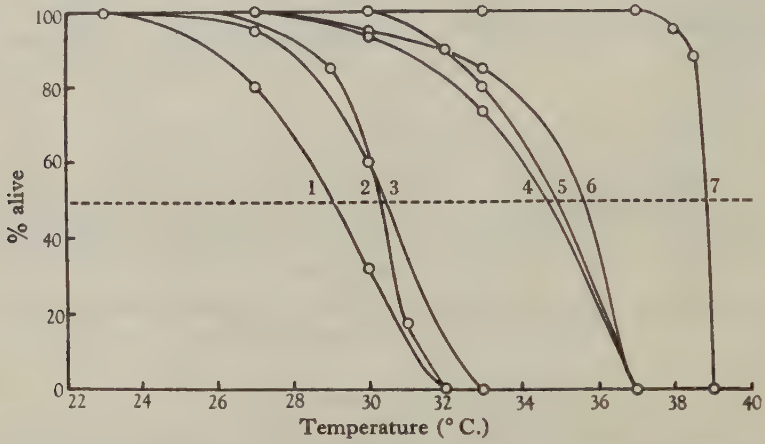

Fig. 3. Thermal resistance of various chironomid larvae. Species used (nos. 1–7) given in Table 6.

(4) DISCUSSION

Of the chironomid larvae whose oxygen requirements have here been studied, the two species from a stream have higher metabolic rates in aerated water than the corresponding species from still water. The similarity in size, structure and activity of the members of the two pairs of larvae eliminates the possibility that the metabolic differences are due to these factors. The fact that relatively high metabolic rates have also been found in stream species of other aquatic arthropods (Fox *et al.* 1933, 1935) suggests that this property may be a general characteristic of animals living in streams. The evidence of Washbourn (1936) that the oxygen consumption of trout fry increases with the current velocity in which they are reared shows that the metabolic difference between fast- and slow-water animals can be acquired during individual life. Although in the case of the trout fry the direct effect of current may well have been the causal factor influencing the metabolic rate, with chironomid larvae, which in their mud tubes are protected from the current, this cannot be so and the significant cause seems more likely to be a high oxygen pressure, if indeed the high metabolism is individually acquired. On the other hand, it may be innate.

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* Whitney (1939) tested this possibility when working with the nymphs of Ephemeroptera. He found the extra survival in oxygenated water as compared with aerated water never greater than 14%, which was not sufficient to obscure the specific differences in thermal resistance.

The high metabolic rate of the stream chironomid larvae is only maintained in fully aerated water, the relation between oxygen concentration and oxygen consumption being approximately a linear one. This contrasts with the non-linear relationship between the two variables in the larvae from stagnant waters. Such differences in animals' responses to low oxygen concentrations have been detected in a wide variety of organisms (Hyman, 1932; Tang, 1933) and have given rise to much speculation as to the general significance of the dependence or independence of oxygen consumption on oxygen pressure. It should be pointed out that the difference between the dependent type of curve shown by the stream chironomids and the independent type of the other chironomids is only a relative one, related to differences in the critical oxygen pressure below which the normal metabolic rate cannot be maintained. This critical oxygen concentration lies between 15 and 44% air saturation of the water in the stagnant water chironomids, and at, or possibly even above, 100% air saturation in the stream ones. That it should differ so markedly in such closely related animals makes the assumption reasonable that it has some special ecological or physiological significance. In the chironomid larvae the differences are related to the habitat of the animals, but Fox *et al.* (1937), measuring the oxygen consumption of various ephemerid nymphs, found that although the nymph with the most dependent type of metabolism (*Baetis* sp.) came from a stream and that with the most independent type (*Cloeon dipterum*) from a pond, the correlation between degree of dependence and habitat did not hold for the other species studied.

The main interest of such comparative studies of metabolism lies in the fact that very closely related and structurally similar animals do show these extensive differences in types of metabolic response to oxygen. In these animals different degrees of dependence cannot be the result of differences in the efficiency of oxygen transport to the tissues, since in the chironomid larvae, at any rate, the size and the speed of blood circulation are the same in both members of the contrasted pairs. The larvae which show dependence are, however, those with a high metabolic rate in aerated water, suggesting a difference in cellular respiratory systems.

The differences in type of metabolism shown by these closely related larvae raises the question of the possible function in respiration of the haemoglobin they contain. Ewer (1942) showed that the haemoglobin of the larvae of *Chironomus riparius* and *C. cingulatus* (two very similar species), while functionless as a transporter of oxygen in air-saturated water, enables them to maintain a normal metabolic rate at lower oxygen concentrations: in other words, by lowering the critical oxygen pressure the haemoglobin was to a large extent responsible for their independent type of metabolism curve. One is probably justified in assuming a similar function of the haemoglobin in the closely allied *C. longistylus*, but the independent curve of *Anatopynia varia* can scarcely be the result of the possession of a functional haemoglobin since the blood of this species contains very little of the pigment. Conversely, the strikingly dependent *Tanytarsus* and *Anatopynia nebulosa* contain as much haemoglobin as *Chironomus longistylus*. In *Tanytarsus* the haemoglobin is only used in oxygen transport at oxygen concentrations below 25% air saturation (Walshe, 1947), at which concentration the larvae, even with the help of their haemoglobin, are only capable of maintaining a metabolic rate less than half that which they have in fully aerated water. To what extent the haemoglobin is really of use to a chironomid with a dependent type of metabolism curve is therefore doubtful: it certainly does not equip them to continue a normal aerobic metabolism at low oxygen pressures.

From an ecological point of view the differences are more easily interpreted. A high metabolic rate and a need for complete air saturation of the water to maintain this rate imply that the stream larvae are only fitted for life in situations where water movement ensures adequate aeration. The oxygen content of the water just above the mud in the brook from which the two stream chironomids were collected was measured at intervals throughout the year and never fell below 78% air saturation, whereas the oxygen concentration in the ditch containing the other two species was always much lower than this. Oxygen samples were taken in this ditch at intervals during one day in summer and even at its highest point the oxygen immediately above the animals' tubes only rose to 58% saturation and throughout the hours of darkness was at the very low value of 2% saturation. The still-water species therefore have to endure very low pressures of oxygen, at any rate in summer, and their ability to maintain a normal metabolic rate at low oxygen pressures fits them for their life in such an environment. It would appear that in such species adaptation to environment has proceeded to enable the larvae to live in habitats deficient in oxygen whereas the stream chironomids are unmodified in this respect, no selection for such an adapted metabolism having been necessary.

With one exception, the larvae of stream chironomids are much less resistant to high temperatures than still-water forms. Whitney (1939) also found that ephemerid nymphs from slow-flowing or still waters had a greater heat tolerance than those from swift streams, and he correlated these differences with greater temperature fluctuations in the former environments. A similar correlation can be made for the chironomids: temperatures in the stream never rose above 15° C. during the summer in which these experiments were made, whereas a water temperature of 20° C. was recorded in the still-water habitats. In general, the thermal resistance of animals is closely related to the temperature conditions of their environment, the maximum temperature for normal life being little above the highest temperature to which the organism is normally subjected, although the maxima can be artificially raised by acclimatization. To what extent the thermal resistance of an animal in its natural state represents an individual acclimatized condition, and to what extent it is the genetical result of adaptation by selection of physiological processes which have temperature optima most fitted to the temperature ranges experienced, is as yet unknown. In this connexion it was interesting to find *Chironomus albimanus* (the stream chironomid with the highest thermal resistance) also living in a stagnant water environment in association with *Chironomus plumosus* and *Anatopynia varia*. Its choice of habitat therefore seems wide and its thermal resistance correspondingly greater than the more typical stream species. This suggests a genetically determined condition.

The maintenance of a normal metabolic rate at low oxygen pressures, and the capacity to withstand both oxygen lack and high temperatures, are features in the physiology of stagnant-water chironomids which enable them to live in such habitats. It is clear that these physiological characters are adaptive, and it is reasonable to suppose that they have been acquired by such chironomids as necessary conditions for life in difficult habitats. On the other hand, because the stream species have a high metabolic rate and are much more susceptible to adverse conditions one may assume that constantly favourable oxygen pressures and temperatures in the stream habitat have made it unnecessary for them to evolve adaptations to meet unfavourable conditions. This does not necessarily imply, as one might be tempted to conclude, that such an environment was the original type to be colonized by chironomids, but rather that stream and pond larvae have evolved along two independent adaptive lines. Adaptation for life in streams demanded modifications mainly in morphology and behaviour as opposed to the profound respiratory specializations evolved by larvae colonizing stagnant habitats.

This investigation was made in the laboratory of Prof. H. Munro Fox.

SUMMARY

1. The larvae of two chironomid species, *Tanytarsus brunnipes* and *Anatopynia nebulosa*, living in streams consume more oxygen than the closely related *Chironomus longistylus* and *Anatopynia varia* from still water.

2. The oxygen consumption of the two stream species falls as the oxygen content of the water diminishes, whereas that of the two still-water species remains approximately constant until the oxygen content has fallen to a low value.

3. Of the larvae of ten chironomid species, those from streams are much less resistant to anaerobic conditions than those from still water.

4. Stream chironomids have a lower thermal resistance than still-water forms.

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ANAPHASE MOVEMENTS IN THE LIVING CELL

A STUDY WITH PHASE CONTRAST AND POLARIZED LIGHT ON CHICK TISSUE CULTURES

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(With Plates 2 and 3 and Thirteen Text-figures)

INTRODUCTION

This paper is an account of observations and measurements we have made on mitosis, and especially on anaphase movement, in living embryonic cells of the chick in tissue culture. We have worked both with polarized light and phase-contrast illumination. Such a study of the normal process in living material is a necessary preliminary to further investigations into the nature and mechanism of mitosis, for although there is a great volume of earlier work, only a small proportion of it concerns the living cell.

There are three studies in the literature on the timing of chromosome movement in anaphase, namely, those of Bělar (1929) on spermatocytes of the grasshopper *Chorthippus*; of Barber (1939) on staminal hairs of *Tradescantia*; and most recently of Ris (1943) on spermatocytes and embryonic cells of certain Hemiptera, mainly the aphid *Tamalia*. Besides measuring anaphase separation, Bělar contributed a theory of the nature of the movement; Barber calculated temperature coefficients for anaphase movement and protoplasmic viscosity; and Ris measured spindle elongation in fixed material, as well as chromosome separation in the living.

The studies on mitosis in polarized light are more numerous, but by no means as detailed. The fullest account is that of Schmidt (1937, 1939) who examined the eggs of the sea urchin *Psammechinus miliaris*, and noticed that chromosomes, spindle and asters were all birefringent. In his book (1937, p. 90), he remarks 'es ist ein wunderbares Schauspiel, den Wechsel des optischen Verhaltens des Kernes in der Folge der Teilungen zu begleiten' and goes on to say that birefringence could almost certainly be found in other cells. Further on (pp. 120–4) he reviews previous observations on mitosis in polarized light. None of the half-dozen or so authors he quotes, however, pursued their observations very far.

By combining the techniques of polarized light and phase-contrast microscopy, we have been able to study both chromosome movement and the changes in the spindle. By recording our results on ciné film we have, moreover, been able to make measurements with some accuracy every few seconds. We have also begun to analyse molecular structure in the spindle and elsewhere by means of polarized light, and have made a first attempt to relate our findings in this direction with the movements we have observed.

This paper is intended to be the first of a series, which, we hope, will deal in more detail with chick cells, marine eggs, and other material. We have put forwa d various ideas in the full realization that it may be necessary to modify or discard them in the light of further discovery.

The responsibility of the authors for the various sections of the work is as follows: polarization microscopy is by M. M. S., time-lapse photography, phase-contrast microscopy and analysis of film material is by A. H. The discussion and the ideas advanced are the joint work of both authors.

MATERIAL AND METHODS

Phase-contrast microscopy

The advent of the phase-contrast method of illumination (Zernicke, 1935; Burch & Stock, 1942) has greatly extended the scope for the study of many types of living cells. In collaboration with Dr H. B. Fell, one of us (A. H.) has used the method in a general study of tissue cultures and an account of the findings is being prepared.

Two versions of the phase-contrast method have been used. In the one, the phase plate has a diametrical etch (Burch & Stock, 1942) and in the other, an annulus. Dr Burch was kind enough to instruct one of us (A. H.) in the preparation and use of the first type, and converted a $\frac{1}{12}$ in. immersion achromatic objective for our use according to his published directions. We owe much more to Dr Burch than any formal acknowledgement can convey. The second type is made by Messrs Cooke, Troughton and Simms, Ltd, and we wish to express our thanks to Mr E. W. Taylor of this firm for his great kindness in lending us one of these phase-contrast outfits.

Phase-contrast illumination can readily be applied to cells in the outgrowth of tissue cultures. The free air surface of the culture, necessary for continued growth, can be retained, although the irregular lower surface of the culture does cause some distortion of the illuminating cone and constant recentring is necessary to keep the system in adjustment when searching a field of cells.

The cultures used were all original explants* of tissues from 9- to 11-day chick embryos, either from the heart, lung, or frontal bone, or from skeletal muscle. Osteoblasts were used for the main part of the work since they have the advantage that the outgrowth consists from the first of flattened cells near, or in contact with, the surface of the coverslip. To reduce the total thickness of the culture chamber to less than that of the normal depression slide culture, a coverslip sealed to a spacing ring of metal was used in place of the normal slide. The dividing cells used for observation were selected from cultures about 24 hr. old, when the mitotic rate is at a maximum.

The first series of mitoses were studied and recorded by phase-contrast illumination, in collaboration with Dr H. B. Fell, whose interest in the method and its possibilities was a factor of great importance in the early stages of the work. Later, Mr L. J. King, Dr Fell's assistant, was able to supply routine cultures of osteoblasts, which have been used for the major part of this work. For Mr King's skill, patience, and ready co-operation, the authors wish to express their warmest thanks.

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* In phase-contrast microscopy, a crowded field reduces the contrast. In original explants the outgrowth is less dense than after sub-cultivation, and the chances of finding isolated cells in division is higher.

Polarized light microscopy

In the summary of his work on the double refraction of mitotic figures in the sea urchin *Psammechinus miliaris*, Schmidt (1937) mentions that the maximum value of the birefringence is about 5 mμ or $\lambda/100$. Path differences of this order can be seen quite readily with an ordinary polarizing microscope, and one of us (M. M. S.) had no difficulty in photographing these figures in the eggs of *Psammechinus* and other marine animals, using an old-fashioned petrological microscope with a tungsten filament light source. (Results not yet published.) However, a study of chick tissue cultures with the same apparatus showed little trace of birefringence in dividing cells. This we assumed to be due to the inadequacy of our technique, and before trying again, we set out to improve the sensitivity of the equipment.

A paper is being prepared by one of us (M. M. S.) on the use of the polarizing microscope to detect extremely weak birefringences, and on the various improvements than can be effected. Here we shall only describe in brief the instrument we used for the present work, which, though capable of further improvement, proved good enough for detecting the main features of the birefringence in chick mitoses.

The instrument we used was not originally a polarizing microscope at all, but an old Zeiss research model, with a rotating stage and a movable iris diaphragm in a swing-out mount below the condenser. To this microscope we fitted a polarizer and analyser of 'Polaroid'. Single thicknesses of 'Polaroid' when crossed transmit a certain amount of purplish blue light which colours the field of a microscope very noticeably. The best samples actually transmit less light at the extinction position than do Nicol prisms (Hallimond, 1944), but the performance of an average pair is not as good. We therefore tried the effect of two layers of 'Polaroid' in both polarizer and analyser. The amount of light transmitted is then greatly reduced; although if the combination is held up to the sun, or some other intense source, a slight purplish blue colour can still be detected. In the microscope, however, the field of view at extinction no longer shows any trace of colour, from which it would appear that the extinction of polarizer and analyser has ceased to be the limiting factor in getting a dark field, and that miscellaneous depolarizations in the optical system are responsible for almost all of the light that gets through the analyser, thus producing a field of view that is grey rather than perfectly black.

The 'Polaroid' we used was obtained from Messrs Watsons, Ltd., mounted between circles of optically flat glass. To double the thickness of 'Polaroid' we simply put a drop of thick immersion oil between the plates, and then pressed them tightly together, making sure that their axes coincided. The plates we used for the polarizer were 1⅛ in. diameter, and these were held in a standard microscope filter holder, fixed below the swing-out mount of the movable iris. The analyser consisted of a pair of $\frac{5}{8}$ in. diameter 'Polaroid' plates held in a rotatable mount threaded with objective screws, which was inserted between the objective and the tube.

We used also a $\lambda/20$ mica plate, made for us by Messrs Cooke, Troughton and Simms, Ltd., which was held just below the substage iris, and could be rotated freely in the iris mounting. The use of such a 'compensator plate' is described at length by Schmidt (1934). Not only is it useful for compensating any stray birefringence in the lenses or slides, but it can be used to measure birefringence, and to increase the contrast of weakly birefringent objects.

Even these measures, however, are not enough to obtain a really good extinction, since even the most accurately plane-polarized beam is partially rotated by refraction at an oblique interface. Between the actual 'Polaroid' sheets in the polarizer and analyser there are some 14–20 air-glass interfaces in the microscope, across which a divergent or convergent beam is transmitted. It is obvious therefore that the possibilities for rotation of the polarized beam are considerable, and the chance of getting a perfect extinction correspondingly slight. The solution is to use as narrow a cone of light as possible, and we therefore stopped down the condenser to an N.A. of about 0·15–0·20, corresponding to an iris diameter of about 5 mm. Theoretically perhaps the objective as well as the condenser should be stopped down, in order to cut out the widely scattered, and hence considerably depolarized light, from the object. This, however, would have the effect of cutting down resolution, and while we found that we were unable to get a field dark enough to detect these weak birefringences with $\frac{1}{12}$th oil immersion objectives of N.A. 1·2–1·3, we could get a reasonably dark field with objectives of N.A. 0·6–0·7. In fact, for all the polarized light microscopy described in this paper, we used a one-sixth achromat of N.A. 0·65.

It will be obvious that with a condenser stopped down to this extent, and with crossed 'Polaroids', very little light is transmitted for observation or photography. The intensity discrimination of the eye falls off considerably with a field darker than about 0·1 f.c., while photographic exposures become impossibly long. We were therefore forced to use an arc lamp as a light source, with an efficient heat filter. The effect of this intensity of illumination on dividing cells is discussed in a later section.

We have already mentioned the use of the mica plate for compensating stray birefringences in lenses or slides. As far as possible, however, we selected components without such residual strain birefringences. Lastly, we were careful to clean all the glass surfaces very frequently. A single piece of birefringent fibre anywhere in the system was quite enough to impair the extinction very seriously.

It is difficult to give precise figures for such a subjective matter as the detection of a weak birefringence. We estimate, however, that with the microscope we have been using, it is possible to measure with reasonable accuracy birefringences down to $\lambda/1500$ (3·3 A.), and to detect them down to $\lambda/3000$ (1·7 A.). It will become apparent later on, however, that even the performance of this polarizing microscope was insufficient to clear up all the points that arose in the course of our observations. Further improvements are needed, and are, we believe, possible.

Apart from the use of an arc lamp to get sufficient light through the system, our technique, both as regards photography and tissue culture, was identical with that already described for phase contrast microscopy.

Those who are not familiar with polarized light microscopy may have some difficulty in understanding parts of this paper. There is an extremely good account in German by Schmidt (1934) of the use of polarized light for biological studies, but there is unfortunately no counterpart in English. Wood (1934) gives a good introduction to polarization phenomena from a physical point of view, and Bunn (1945) gives a more detailed description.

Suffice it here to say that a polarized beam of light is retarded to differing extents in different axes when passing through any ordered molecular structure. On emerging again the two mutually perpendicular components of the light are thus slightly out of step, and the beam is said to be 'elliptically polarized'. The extent to which they are out of step, i.e. the path difference, is the measure of the strength of birefringence. A proportion of such an elliptically polarized beam is capable of passing through a prism or polaroid set at right angles to the original polarizer, while light that has not been elliptically polarized, is stopped. A birefringent, ordered structure thus shows up white (in the case of weakly birefringent objects such as those we are dealing with) on a black background. The compensator plate mentioned earlier can be used to enhance this contrast of white on black, or to reverse it so that the birefringent object appears black on a white background. Photographs compensated in both ways will be found in this paper.

Photographic recording

Without some means of taking serial photographs of a cell in division, it would not be possible to make more than a few rough measurements of the progress of an anaphase as rapid as that of the chick. Permanent records on ciné film make it possible to re-examine the process as fresh lines of inquiry are suggested, and to study both individual pictures, and continuous film in speeded-up motion. The two methods are complementary, and we turn repeatedly from one to the other in building up a picture of the living process.

We have used substandard 16 mm. film rather than standard 35 mm., because of the shorter exposure needed with a small initial magnification (up to × 100). This is essential in polarized light microscopy, where light is the limiting factor. Our films are generally of sufficient photographic quality for the purposes of research, and the best of them when projected are adequately detailed and sharp. When, however, paper enlargements are prepared from them at a magnification of 5–10 times, the grain of the film is very evident, and for this reason the plates which illustrate this paper do not indicate the true capabilities of phase-contrast illumination in the photography of living cells.*

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* We have, however, included two phase-contrast photographs from normal-sized negatives, which are more representative. Fig. 3 of Pl. 2 is of a locust primary spermatocyte in metaphase, and illustrates the way in which detail at different levels in a cell can be separately resolved (preparation by Miss A. Martin).

For phase contrast work alone, emulsions of great speed are unnecessary. However, if a fast film is used, then the illumination can be cut down to levels even below that necessary for ordinary visual observation. The Burch system of phase contrast, with the narrow diametrical etch, gives a high general contrast, so that a low contrast fine grain film gives satisfactory results. The annular system on the other hand, while having various advantages, gives less general contrast than does the Burch system. For ciné micrography with the annular phase plate, an emulsion of high contrast is therefore advisable, and for the best results higher initial magnifications on 35 mm. film should be used, in order to overcome the troubles of 'graininess'.

For polarized light ciné-micrography, the fastest possible emulsion is needed. Such emulsions are always grainy, and at the low initial magnifications we have had to use, this has been a source of some trouble. We have found Super XX and 'Fluorodak' to be the most suitable; the high contrast of the latter is especially valuable.

We have used two 'time-lapse' cameras in which warm-stage microscopic systems have been incorporated. Both instruments have been designed, and largely constructed by one of us (A. H.). In one, the maximum speed at which exposures are made is eight to the minute. This, though sufficient for analysis of single frames, gives too great a speeding-up in projection. The other apparatus can take up to thirty exposures a minute. In both, it is possible to focus the microscope in between exposures. This is an essential feature, although adjustment should be restricted to the minimum. Considerable practice is needed to maintain focus when the image is seen in the viewing piece for only 1–2 sec. between exposures. It is proposed to describe the mechanical and electrical devices used in these cameras in a subsequent publication.

The processed films were studied in projection and in frame-by-frame analysis; both are necessary to appreciate the details of the movements in cell division. In analysis frame-by-frame, two procedures were employed. In one, the film was viewed in a low-power binocular magnifier, and measurements made directly with an eyepiece graticule. The other method was to project each frame on to a table, and make serial drawings of the details required. This method is essential where a number of relative measurements are needed.

Biframe recording

This term we have applied to a method of alternate serial photography by the two optical methods. In pictures of dividing cells of the chick taken by polarized light, the chromosomes are not visible; if they are anisotropic, their birefringence is below what we can at present detect. On the other hand, in phase-contrast pictures the spindle is invisible, and so to study the relations between chromosome movement and the changes within the spindle, both sets of data must be obtained from the same cell.

In order to change from one method of illumination to the other the following method was adopted. A Cooke, Troughton and Simms phase-contrast objective of

× 40 magnification was used throughout. In the substage system, there is mounted in a plane near that of the iris diaphragm a sector plate which can rotate through an arc of about 20°. At one extreme of its traverse, the plate allows a central beam of polarized light through the condenser, which passes through the objective phase plate well inside the inner circle of the internal margin of the annulus. At the other limit, the plate presents an annulus whose image in the plane of the phase plate coincides with the phase ring in the normal manner. Thus the change from one system of illumination to the other is effected by a simple movement of the sector plate, actuated by an electromagnet, and controlled by a mercury switch incorporated in the timing gear of the camera system.

On the phase-contrast image, there is superimposed one in polarized light, but the latter is not prominent, owing to the depolarization of the light at the larger aperture of the phase-contrast annulus.

The exposure necessary for the two sets of pictures is not the same. At an initial magnification of about × 100 on the film, the exposure was $1\frac{1}{2}$ sec. for phase contrast, and for polarized light about 4 sec. To achieve this, the timing gear was modified to interrupt the camera drive during the polarized light exposure, and an external controller restarted the camera motor after the necessary interval had elapsed. In this way, one pair of pictures was taken every 9 sec. The film used was 'Fluorodak', which has the advantage of a high speed, with a considerable contrast.

The negatives were analysed in the usual manner, and the positive was projected in a special projector which shows two pictures on consecutive frames simultaneously. This projector was modified from a standard size machine, by fitting a special set of sprockets, and a 16 mm. gate, with an opening for the size of two frames.

The difficulties of adjustment in biframe micrography are formidable, and it proved advisable to minimize the necessity for centring by making contact preparations of the cultures immediately before use. The effect of this on cells in division is discussed in a later section.

VISUAL OBSERVATIONS

The general appearance of dividing cells in phase-contrast illumination

In a living chick cell which is about to divide, the first detectable change is the dissolution of the nucleoli, which takes place surprisingly rapidly, and is complete within a minute. This rate is faster than that noticed by Lewis (1940) in rat fibroblasts. The chromosomes at this stage are not yet visible, and the nuclear area is a uniform grey tone. The nuclear membrane disappears at about the same time as the nucleoli.

Within the next 5 min the chromosomes gradually become visible, and are at first arranged in radial threads. They show movement, but not, we think, on the scale of Strangeways' 'eels in a box' (Strangeways, 1922). They can be seen in projected films in an irregular quasi-Brownian movement. On this is gradually imposed the concentration into the metaphase plate. The irregular motion of the chromosomes continues during metaphase, as Lewis (1939) has described. By this stage, the contrast between chromosomes and cytoplasm is fully developed, and the cell has withdrawn most, though generally not all, of its processes, and is partially rounded in form.

The cell inclusions concentrate round a spherical or ovoid surface which bounds the nuclear area. The filamentous mitochondria of the resting cell shorten to thick rods, sometimes branched, which envelop the nuclear area, along with granular inclusions. This boundary surface is sometimes also seen in polarized light pictures.

The spindle itself cannot be seen by phase contrast illumination. Sometimes however, thin mitochondrial threads can be seen extended longitudinally inside the spindle.

The metaphase plate is often first formed at an oblique angle to the equatorial plane, and sometimes even parallel to the long axis of the cell. It then gradually rotates to its transverse position. This point will be discussed further in a succeeding section.

The irregular chromosome movements of metaphase often obscure the precise moment at which anaphase begins. The impression gained from observations is that often there are one or more 'false starts'. It almost seems as if an equilibrium between opposing forces is maintained, until the balance is finally lost at anaphase.

The form assumed by the chromosomes early in anaphase is very variable. Often the daughter chromosome groups are bowed with concave sides facing each other. Sometimes the chromosomes are drawn out into two or more longitudinal masses, which only reform into two coherent groups of daughter chromosomes late in anaphase. Other irregularities in anaphase movement, revealed by analysis of film records, will be discussed later.

The first sign of the approach of cell cleavage is that granular material at the cell equator invades the spindle area, and constricts the interzonal region. The cleavage furrow then appears and gradually deepens.

We agree with Chambers (1938) that the characteristic blebs of anaphase bubbling are less evident in the equatorial region. The amount of bubbling is very variable indeed. It is by no means confined to late anaphase and telophase, but is prominent at all stages of division in myoblasts, where telophase is so violent that the point of cleavage is usually obscured.

After telophase the daughter cells gradually flatten, and extend processes into the surrounding medium. Nucleoli and nuclear membranes are usually visible within 10 min. of cleavage.

The process of division is illustrated by phase contrast in Pl. 2, fig. 1.

General appearance of cells by polarized light

A tissue culture in the field of a polarizing microscope of sufficient sensitivity has a most striking appearance. Not only are the mitotic figures birefringent, but the cell walls, the general cytoplasm and even the plasma gel around the cells all show signs of orientation. Very often groups of cells grow out parallel, in which case spindles, cell walls, cytoplasm and plasma all stand out bright at the same time, giving a remarkable impression of the way in which the molecular form of these structures is related to the axis of growth. One such case is shown in Pl. 2, fig. 6.

Cell walls are very often, if not always, birefringent in side view. They have been the object of extensive study, and our knowledge of their optical properties is well summarized by Harvey & Danielli (1938). The walls in these particular cells were negatively birefringent in the radial axis (considering the cell as an elongated cylinder). This indicates that they are, predominantly at least, composed of protein material spread in the plane of the surface. Their observed path difference was very variable, with an average value of about 5 A. ($\lambda/1000$).

Apart from the observations of Pfeiffer (1942) on frog eggs and of Schmidt (1937) on the living axopodia of *Heliozoa* and *Radiolaria*, birefringence has very seldom been observed in ordinary living protoplasm. Birefringence of the cytoplasm in the fibroblasts which we studied was always most apparent in elongated cells, and especially in the long thin processes characteristic of inter-mitotic cells. It was usually absent, or at least not detectable, in cells undergoing mitosis. The birefringence was always positive with respect to the long axis of the cell, showing that the protein chains were oriented predominantly parallel with that axis. It was very variable in its strength; we noticed path differences from 10 A. ($\lambda/500$) down to about $\lambda/3000$ or the limit of detectability, with an average of perhaps $\lambda/1000$ to $\lambda/1500$. Since these processes are known to be considerably less than 1 μ thick, the protein material in them must be in a high state of orientation (cf. footnote on p. 67).

In addition to the general cytoplasmic birefringence, positive with respect to the long axis of the cell, there is quite often to be seen a roughly circular patch, *negatively* birefringent with respect to the long axis. These patches are usually near one end of the main body of the cell, and seem to be more prominent in mitosis, though they are sometimes visible at other times. Such a patch of negative birefringence in the midst of positive birefringence is surprising. If it consists of normal protein, then the chains must be orientated at right angles to all the rest. Alternatively, it might be composed of nucleic acid, or nucleic acid and protein, oriented in the long axis of the cell. Such a patch is shown in Pl. 3, fig. 2, throughout the sequence.

The birefringence to be observed in the plasma medium on which the cells are cultivated is often stronger than that in the cells themselves. The medium is normally perfectly isotropic, but for distances up to 5 and 10 μ from cells it may show a path difference of as much as $\lambda/250$. The amount and the extent of the birefringence is very variable; it is usually only to be seen around elongated cells, and our impression is that it is to be correlated with active growth outwards from the explant. Whether it is a result of the movement, or of the metabolism of the cell, is not clear. The birefringence is positive with respect to the long axis of the cell, showing that protein material is being oriented parallel with that axis. The experiments of Weiss (1934) are of interest in this connexion.

The first sign of a spindle in early metaphase is a small patch, more or less circular, which from the beginning shows much the same degree of birefringence as the full metaphase spindle. In Pl. 2, fig. 2 are illustrations selected from a series of photographs taken at 8-sec. intervals; in the first of these there is no sign of a spindle, while in the second a distinct body, some 2μ in diameter is to be seen. This circular patch grows rapidly and soon assumes the typical spindle shape.

The birefringence of the fully developed metaphase spindle has an average value of about 10 A. ($\lambda/500$), and is usually greatest at the poles, although the spindle is only about one-fifth as thick there as at the equator. The significance of this is discussed later.

Soon after anaphase begins, the spindle starts to elongate. The birefringence in the equatorial region drops, but rises again later as the interzonal region begins to constrict in cleavage. At the poles, birefringence persists well into telophase.

The spindle is not the only birefringent element of the mitotic figure. At either pole, radiating out from the centrosome, is a small structure which can only be described as an aster. The part of this structure in line with the spindle is positive with respect to the axis of the spindle. The part radiating out at right angles has the opposite sign. Such an appearance indicates a solid radiate structure. These asters are by no means commensurate in size with the spindle, being only 2 or 3μ in diameter, and very feebly birefringent. They can just be made out in Pl. 2, fig. 4. Their similarity to the asters to be seen in sea urchin eggs is obvious (Pl. 2, fig. 5).

It remains to describe the birefringence phenomena of cytoplasmic cleavage. Most striking is the birefringence of the cytoplasm within the constricting neck. The constriction starts at the equatorial region of the spindle, which by this stage is rather weakly birefringent. As the constriction develops, birefringence in the neck increases. When the two cells are nearly separate, this narrow neck may be as strongly birefringent as was the whole of the original metaphase spindle ($\lambda/500$) and like the spindle, is positive with respect to the long axis, indicating protein orientation in that direction. Since the neck is only about 1μ thick at most, it must be fairly completely oriented. Ultimately the strand appears to snap, and the birefringence fades away. (See Pl. 3, figs. 1 and 2). This secondary birefringence in the spindle region is perhaps comparable with the phragmoplast of plants (Darlington & Thomas, 1937).

The actual constricting collar is also birefringent. In Pl. 3, fig. 1 the collar appears as two V's, lying with their points inwards, and showing black, as opposed to the spindle and cell walls which are bright. The arms of the V's are, however, more or less at right angles to the long axis of the cell, and would therefore show up in the opposite sense if they were in fact of the same composition as either the cell walls of the spindle. Lastly, we may notice a phenomenon illustrated in Pl. 3, fig. 1, minute 9, which usually appears when the cells are almost fully separated. Lying perpendicularly across the birefringent neck there appears a line, bright or dark in the same sense as the neck. This is puzzling, since a number of protein chains would hardly be expected to form themselves into such a transverse structure, while remaining oriented perpendicular to its long axis. Further observation is needed before we can interpret these details of the cleavage process with any certainty.

RESULTS FROM PHOTOGRAPHIC RECORDS

Measurements of anaphase movement

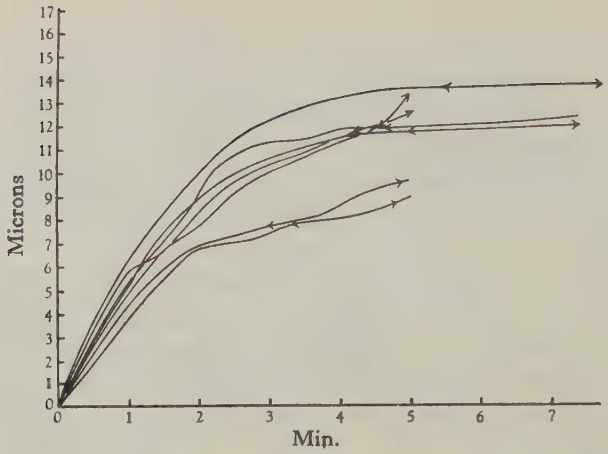
Text-figs. 1–3 represent three sets of curves of chromosome separation in anaphase. Text-fig. 1 is of seven myoblasts and Text-fig. 2 of eight osteoblasts; both these sets are from phase-contrast records, where the intensity of light employed was as low as possible.

The curves are all of the same general form, and the minimum final separation of the daughter chromosome groups is approximately the same in each group, namely 9–10μ. The maximum for the osteoblasts, however, is greater than that for the myoblasts. In both groups, the smaller examples tend to be from cells isolated in the medium; the larger are typical of a crowded field of extended cells in parallel arrangement.

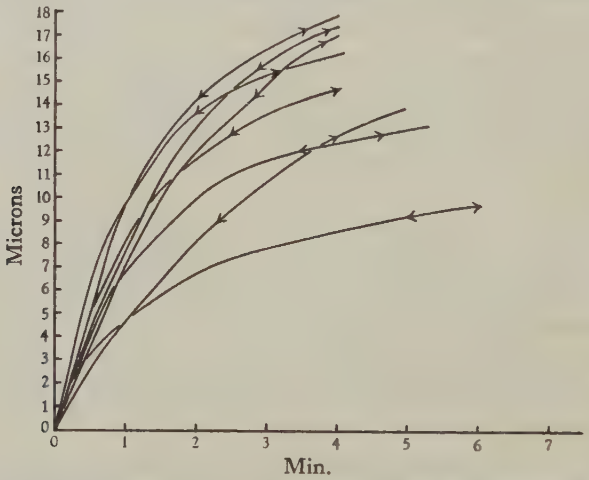
The time of cleavage is shown by two points marked on each curve: the first is approximately that at which the cleavage furrow and the interzonal constriction of the spindle first appear; the second denotes the stage when cleavage is near completion, and the daughter cells are connected by a narrow interzonal thread. It will be seen that myoblasts cleave much later in anaphase than do osteoblasts. In general, cleavage progresses more slowly in cells which begin late; but in myoblasts the end stages are often obscured by the vigour of the bubbling process.

Text-fig. 3 shows the set of anaphase curves of osteoblasts from the series of biframe recordings. Text-fig. 4 gives the averages obtained from Text-figs. 1–3. These are introduced to show the effect of the observational procedure. It was mentioned above, that in addition to exposure of the cells to the strong light required for polarized light micrography, it was necessary on optical grounds to remount the cultures as contact preparations; that is, with the lower surface of the culture in contact with a plain glass slide. This subjects the cells to a temperature shock, to which they are known to be sensitive. It is evident from Text-fig. 4 that this procedure does not alter the shape of the anaphase curve, but does result in a decrease of about 2μ in the final separation. There is, however, in most examples a marked slowing down of cleavage, although in one instance a cell divided earlier than any other periosteal fibroblast we have seen.

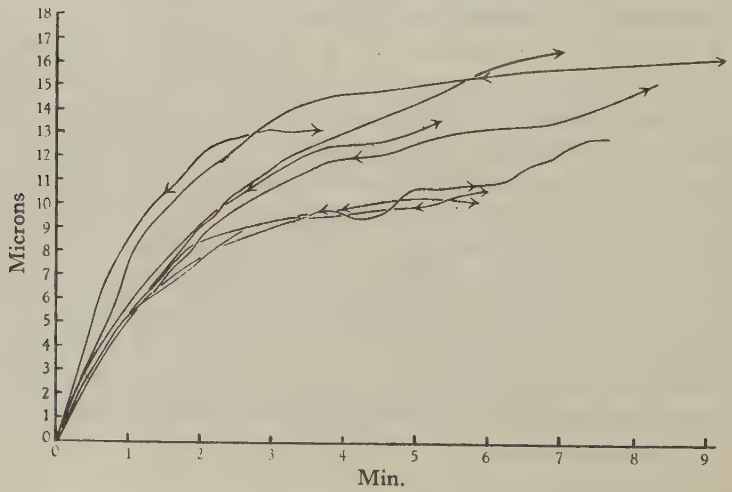
Text-fig. 5 shows a series of six curves of spindle length in osteoblasts from polarized light recordings, without the 'biframe' procedure. These cultures were not used as contact preparations and therefore show the effect on cleavage of strong light alone. Although in a polarized light record it is difficult to determine the point at which anaphase begins to within a half minute, the curves of Text-fig. 5 have been superimposed as accurately as possible. The time at which cleavage begins is marked on the anaphase portion of the curves, and that at which the birefringent interzonal strand is apparent is also indicated, although at that stage the rest of the spindle is often no longer visible. Text-fig. 5 shows that in this group cleavage begins 2–3 min. after anaphase, and is completed within 2–3 min. This is a slightly longer period than that occupied in cleavage by the osteoblasts illuminated less strongly, but is shorter than for those cells which, in addition, were used as contact preparations.



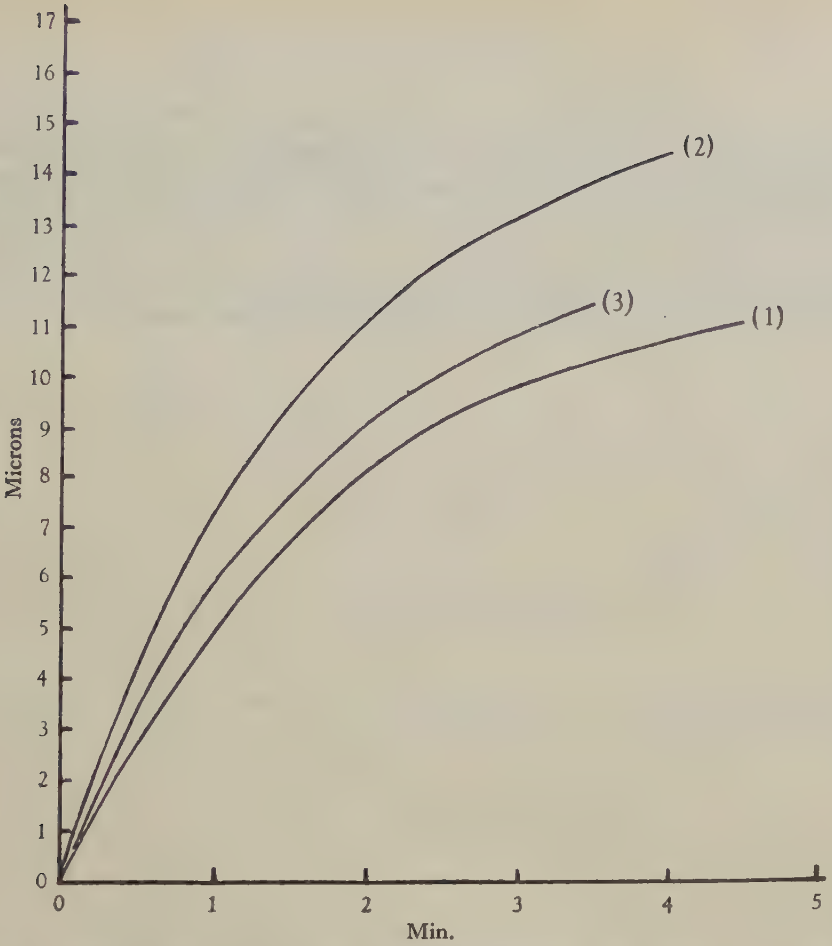
Text-fig. 1. Chromosome separation curves of a group of seven myoblasts, from phase-contrast records. The duration of cleavage is indicated on each curve.



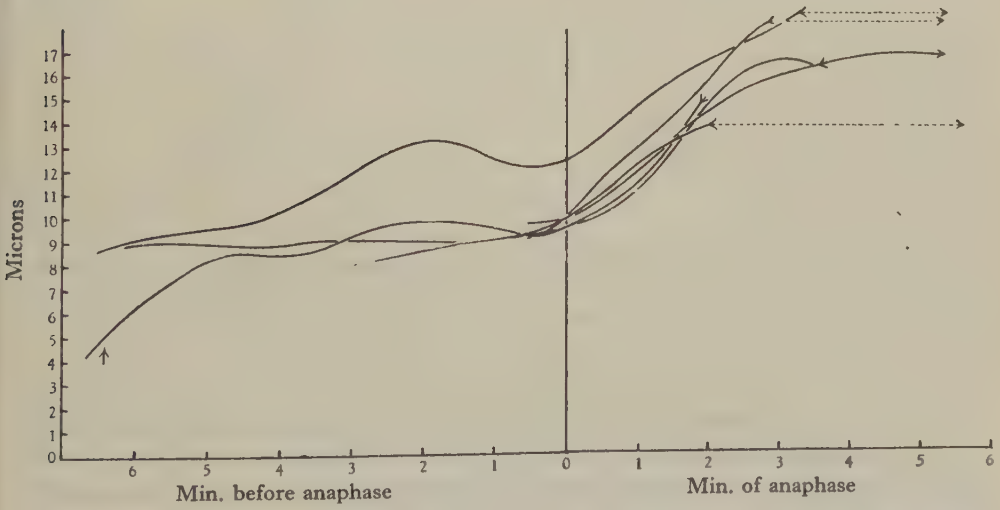
Text-fig. 2. Chromosome separation curves of a group of eight osteoblasts, from phase-contrast records. The duration of cleavage is indicated on each curve.



Text-fig. 3. Chromosome separation curves of a group of eight osteoblasts, from 'biframe records'. The duration of cleavage is indicated on each curve.



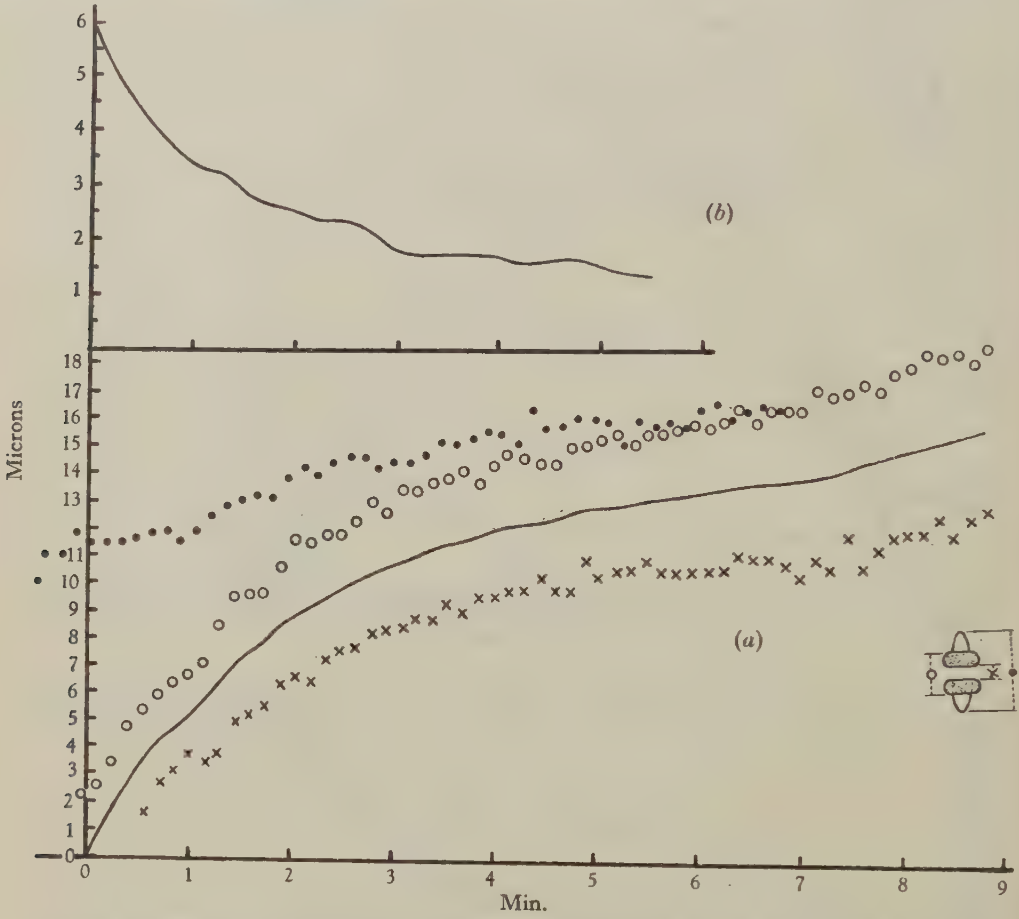
Text-fig. 4. Average curves obtained from the groups of chromosome separation curves respectively of Text-figs. 1–3.



Text-fig. 5. Curves of spindle length of a group of six osteoblasts from polarized light records, both during metaphase and anaphase. The duration of cleavage is indicated on each curve. The vertical arrow points to the record of a sequence from which the photographs of the origin of a spindle (Pl. 2, fig. 2) are taken.

It is apparent therefore that the observational conditions affect cleavage much more readily than chromosome separation. Both strong light and temperature shock do, however, exert some effect on the magnitude of anaphase movement, although the resulting curves are exactly similar in shape to those of ordinarily treated cells.

In Text-fig. 6 is the full set of data obtained from a biframe record. Text-fig. 7 shows the average curves obtained from eight sets of biframe data.



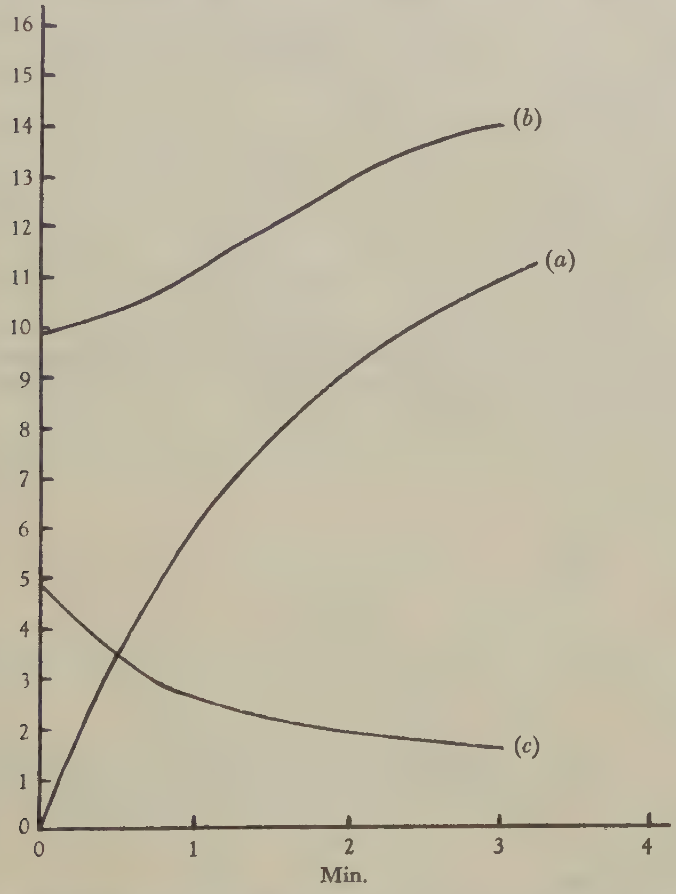
Text-fig. 6. Data obtained from a single biframe record, namely that of Pl. 3 (*b*). In (*a*) spindle length and chromosome separation are plotted, according to the conventions given. In (*b*) the length of one set of chromosome fibres (the distance from the chromosomes to the spindle pole) is plotted.

Subsidiary chromosome movements

In addition to the normal sequence of chromosome movement, we have observed certain subsidiary motions, both in the metaphase plate and in anaphase. These are interesting and possibly significant in interpreting the mitotic process, and further reference will be made to them in the discussion.

(1) *Longitudinal movements*

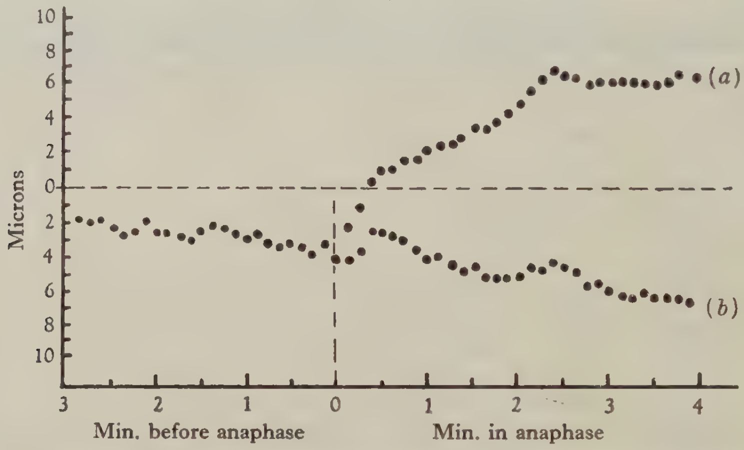
Lewis (1939) has described an irregular longitudinal oscillatory motion of metaphase chromosomes in dividing cells in tissue cultures. Such movements, in our experience, are always present at this stage of division. They are sometimes rhythmic and one has the impression from the projected film of repeated attempts at anaphase, the last of which is successful. Lewis explains the movement as the result of varying tension in the spindle fibres to which the chromosomes are attached. This may well be true, although his relation of 'viscosity' to tension in a solid fibre seems obscure. Some of our films of cell division give the impression that a part of this longitudinal movement may be due to oscillation of the whole metaphase plate in the plane of the culture.



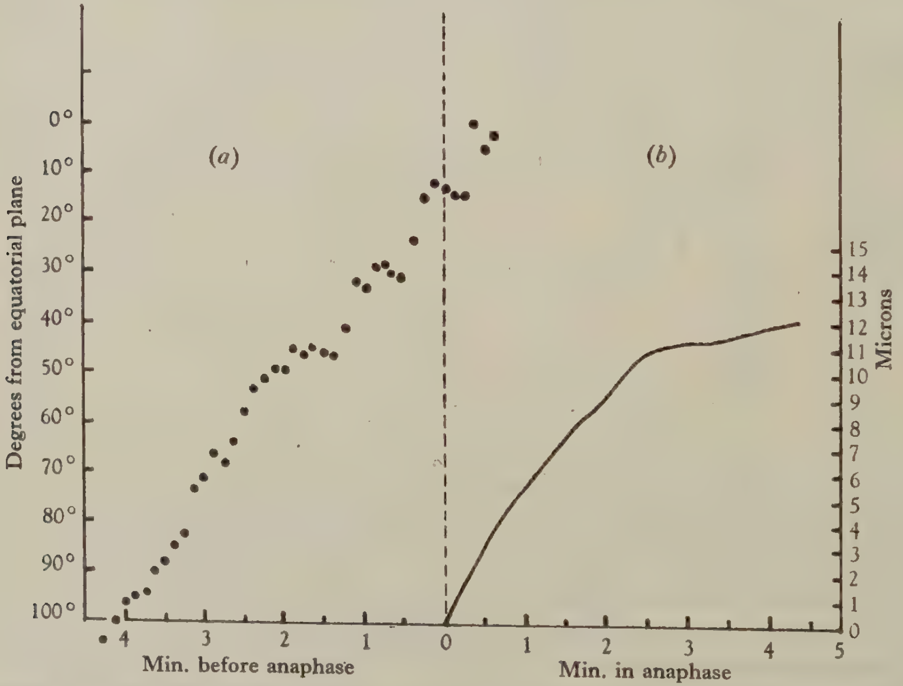
Text-fig. 7. Average curves of (*a*) chromosome separation, (*b*) spindle length, (*c*) length of one single set of chromosome fibres obtained from six sets of 'biframe' data.

In anaphase we have noticed another type of subsidiary longitudinal movement, which may be termed the 'asymmetrical jerk'. Several instances of this have occurred. It can be illustrated by instancing the division of an extended myoblast.

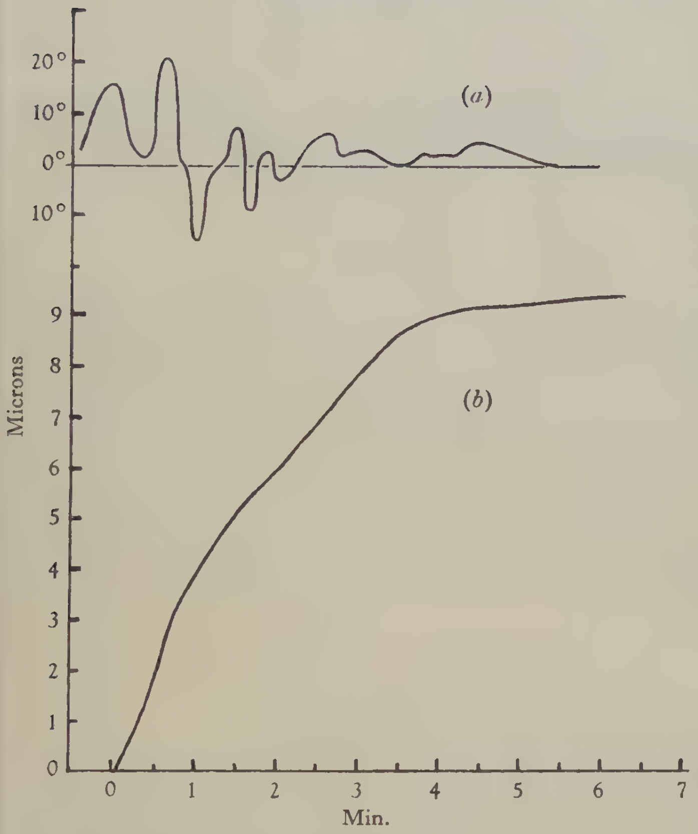
In Text-fig. 8, the distances of chromosome groups from a central reference point measured along the axis of the cell are plotted against time. Such measures plotted through metaphase and anaphase would result in a symmetrical Y-shaped figure if the daughter chromosome groups separated with the same velocities at each instant.



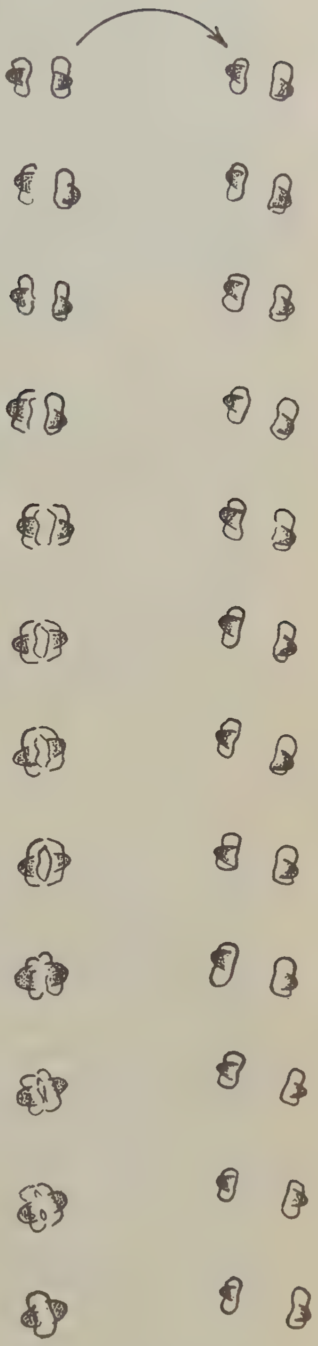
Text-fig. 8. Distances of metaphase and anaphase plates from a fixed point of reference of a myoblast in division, to illustrate the 'asymmetrical jerk'. During the first ½ min. of anaphase, chromosome group (*b*) moves in the 'wrong' direction.



Text-fig. 9. (*a*) Rotation of the metaphase plate of a myoblast in division to the final equatorial position as indicated by the angular co-ordinate. (*b*) Chromosome separation curve of the ensuing anaphase.



Text-fig. 10. (*a*) Oscillation of the metaphase and anaphase plates in an osteoblast in division. 0° represents the equatorial plane. (*b*) Chromosome separation curve of this division.



Text-fig. 11. Tracings from the anaphase of a 'biframe' record of spindle and chromosome groups where each shows independent oscillation. 9 sec. intervals; × 1180.

Text-fig. 11.

In this particular anaphase, they do not, and the resulting asymmetry is most marked at the beginning of anaphase when daughter chromosome group (*b*) for 23 sec. actually moves the wrong way. It was established, by comparison of successive frames, that this asymmetry was not due to displacement of the whole cell in the photographic field.

(2) *Rotation in metaphase*

This has been seen so far in three cases, of which two were in extended myoblasts. One of these is recorded in Text-fig. 9. The metaphase plate first appeared at an angle of over 100° to the equator. Text-fig. 9 describes the rotation of the metaphase plate to the normal position.

(3) *Oscillation in anaphase*

This type of movement is quite common; the chromosomes in early anaphase swing through a few degrees on either side of the equator. Text-fig. 10 records an instance of this oscillation occurring to an unusual extent. The particular cell was an osteoblast. Some irregular angular motion occurred in metaphase; but after 15 sec. of anaphase, the separating chromosome groups made three almost regular oscillations, of diminishing amplitude.

Text-fig. 11 refers to the first part of anaphase of a 'biframe' record. The outline of the chromosome groups and of the spindle in each pair of frames are superimposed. It can be seen that both spindle and chromosomes oscillate to some extent independently of each other.

DISCUSSION

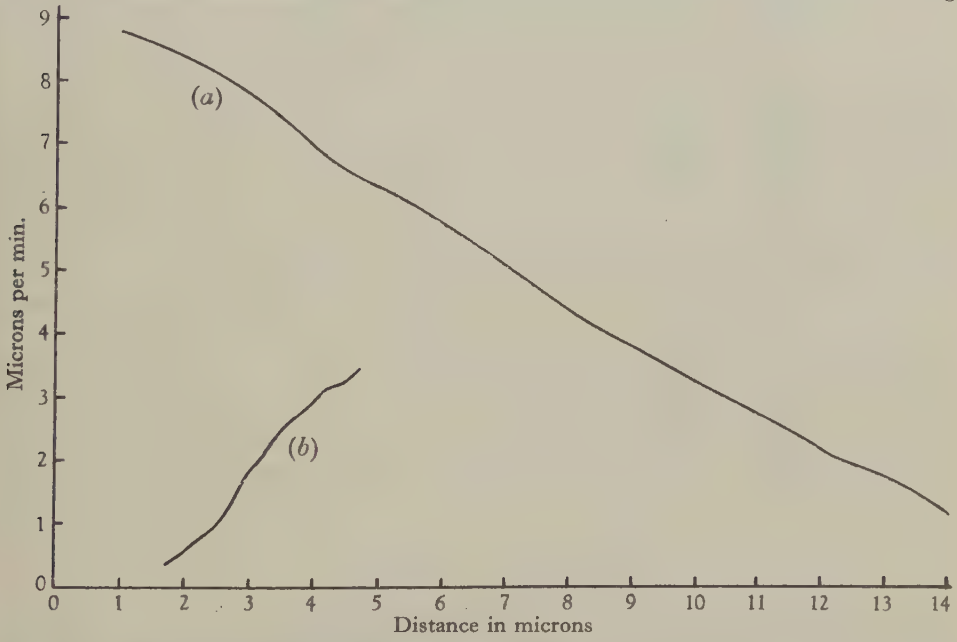
Evidence from anaphase movement curves

The first set of time-lapse photographs, by phase-contrast illumination alone, gave curves varying in magnitude, but of rather similar shape. The averages of these curves are given in Text-fig. 4. They show that the velocity is greatest when the distance between chromosomes is least, and that it falls off steadily as the distance increases. This is compatible with a hypothesis of mitosis involving either repulsion between chromosomes, or a contractile mechanism operating from the centrosomes. It does not seem to us compatible with a hypothesis involving forces of attraction between centrosomes and chromosomes, such as those of Zirkle (1928) and Kuwada (1929); for in this case, the velocity should increase as the chromosomes separate and move nearer to the poles. We may therefore provisionally rule out attractive forces as far as anaphase is concerned, and go on to consider whether the observed movements can be related to any of the other main theories of mitosis.

If chromosomes were large objects moving under the influence of ordinary forces, we could deduce with some certainty from curves of motion the nature of the forces at work. In fact, however, chromosome mechanics cannot be studied with any great rigour, and we are forced to make a number of assumptions.

Since the chromosomes of the chick are extremely small objects (1μ diameter or less) and since the forces acting on them must be large in proportion to their weight, we may reasonably assume that they will reach their terminal velocity extremely quickly under the influence of any force. Moreover, since their absolute velocities are very small (4μ per min. or $\frac{1}{4}$ mm. per hr.) we can use Stokes's formula, in which the terminal velocity of a particle is related linearly to the force acting upon it. From the velocity of the chromosomes we can therefore derive the variation with distance of the force acting upon them, and so, perhaps, deduce something of its nature.

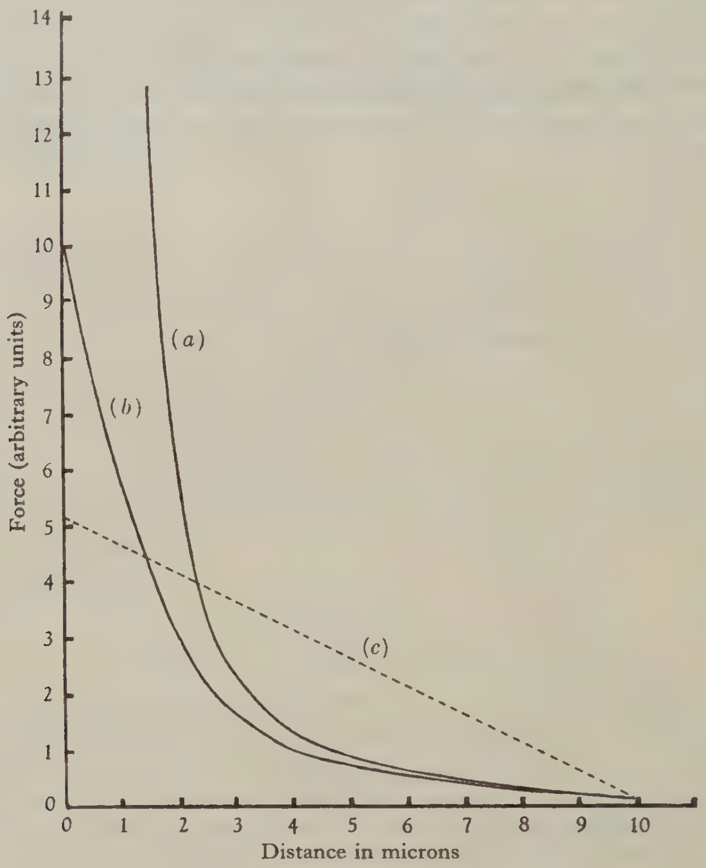
Two objections can be raised to this procedure. The first is that we should derive our estimate of force from acceleration in the ordinary way. But the inertia of a chromosome must be negligible; while in any case, as will appear later, the graph of chromosome separation against time, from which we derive much of our argument, is an exponential, so that the velocity and acceleration graphs are of the same form. The second objection is that Stokes's formula cannot be applied to a non-Newtonian fluid such as protoplasm. This, in general, is perfectly true. We are not, however, concerned with the forces needed to overcome the initial rigidity of protoplasm, but with the forces involved, once movement is established. Moreover, we are concerned only with very small absolute velocities, and with a very small range of these velocities. As long as the approximate nature of our assumptions is realized, there is little danger in making use of them in order to render the problem amenable to analysis.



Text-fig. 12. Velocity plotted against distance for (*a*) the average chromosome separation curve from the data of Text-fig. 2, (*b*) the chromosome fibre shortening curve of Text-fig. 7 (*c*). The velocity of a single chromosome group is half that given in (*a*).

When the slope of the mean chromosome separation curve, representing velocity of chromosome separation, is plotted against the distance between chromosome groups, the graph of Text-fig. 12 (*a*) is obtained. This graph is a close approximation to a straight line, indicating that the original distance-time relationship is exponential.*

If we treat individual pairs of chromosomes, or whole groups of chromosomes as simple charged spheres, the force between them would vary as an inverse square law, producing a force-distance graph of the type of (*a*) in Text-fig. 13. But, if, as may be more reasonable, we treat the groups of chromosomes as charged plates, the position is more complicated. They will still repel on an inverse square law at large distances; though when extremely close together, the repulsive force will be more or less independent of distance. At intermediate distances, the force will change from a constant to the inverse square relationship. Such a graph is shown



Text-fig. 13. Theoretical velocity-distance curves, based on inverse square law electrostatic repulsion force. (*a*) Between charged spheres; (*b*) between charged plates. The dotted line (*c*) represents the type of relationship actually obtained.

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* For the first few seconds of anaphase, the measurement of chromosome separation is uncertain. By the time that chromosome movement is measurable with any accuracy, the maximum velocity has been attained. The velocity-distance curve, drawn in Text-fig. 12 (*a*) as an approximately straight line descending from an initial maximum at about 6 sec. must in reality show a rapid rise from the origin to this maximum.

at (*b*) in Text-fig. 13. Graph (*c*) in Text-fig. 13 represents the type of relationship actually observed. (*a*), (*b*) and (*c*) are plotted with a common point, representing equal forces at a distance of 10μ apart, so that the variations of force with distance can be more readily followed.* Even allowing for large errors, it would be difficult to confuse curves of the type of (*a*) and (*b*), derived from theories of repulsion, with curve (*c*), which represents the observed relationship.

Although there is some variation among the anaphase curves obtained by other workers on other types of cell (Bělar, 1929; Barber, 1939; Ris, 1943), it appears that none are any more consistent than ours with inverse square repulsions. While therefore an electric charge on the chromosomes, by whatever means it be produced, does not seem likely to bring about the observed anaphase movement curves, there are a number of other possibilities to be considered.

The so-called 'long range' electrical forces of London (1942) and others have been mentioned as possible agents of chromosome movement (Barber & Callan, 1943). These forces do not fall off with distance in the simple manner of an inverse square, and might perhaps produce the observed results. At present, however, very little is known of them.

Diffusion processes might also give rise to a relationship of the type we have found, and the velocities observed only demand concentration gradients of quite small magnitudes (Rashevsky, 1938).

While, therefore, theories of anaphase based on diffusion or 'long range' forces between chromosomes might fit in with the observed movement curves, there are other powerful objections which can be raised against such theories. These objections are dealt with by Schrader (1944), while Cornman (1944) goes on to show that only some sort of traction fibre theory can overcome them. His arguments seem to us cogent, and we shall therefore examine in some detail this last remaining hypothesis in the light of our findings.†

The force exerted by an extensible object which obeys Hooke's law is proportional to the extension. Supposing therefore that a tiny spring were attached at one end to a chromosome, and at the other end to a point in the cytoplasm corresponding to the observed final position of the chromosome, we should expect exactly the equation that we do, in fact, find. Biological contractile mechanisms do not obey Hooke's Law at all closely, though they usually approximate to it at normal extensions. The linear relationship is therefore genuinely suggestive.

Assuming that a contractile mechanism operates from a point at, or very near, the final position of the chromosome groups, it seems almost certain that it must operate from the centrosome. Herein, however, lies a difficulty: for the spindle, at either end of which lie the centrosomes, lengthens during anaphase (Text-fig. 7). And the primary measurement to be considered in any contractile hypothesis is not the distance between chromosomes, but the length of the chromosome fibres. If the spindle remained constant in length, the distance between daughter chromosome groups would give a measure of chromosome fibre-length. But when the spindle is elongating at the same time as the chromosomes separate, this is not the case. The linear relationship we have discovered is therefore either a mere coincidence, or, being derived from two separate movements, cannot immediately be interpreted.

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* Curve (*a*) is simply that of a normal inverse square relationship, i.e. $F=\frac{k}{d^2}$ where F is the force, k is a constant, and d is the distance apart of the repelling spheres. Curve (*b*) is constructed from the usual formula for the force between charged plates, i.e. $F=c\left[1-\frac{d}{(d^2+r^2)^{\frac{1}{2}}}\right]$ when F is again the force, c is a constant, d is the distance apart of the plates, and r is their radius.

† This is not to deny that other forces, perhaps electrical, may be at work at other times in the mitotic cycle. The evidence simply indicates that such forces are not important in the anaphase of the chick.

Average curves of chromosome separation and spindle lengthening taken from biframe data are shown in Text-fig. 7 (*a*), (*b*). Spindle length minus chromosome separation, then, gives the length of the two sets of chromosome fibres. The change in length of the single set of chromosome fibres is shown in Text-fig. 7 (*c*). It is evident at once that most of the movement of chromosomes on the spindle occurs in the first minute or two of anaphase. Subsequently, there is little movement on the spindle, but a considerable elongation of the spindle as a whole. In consequence, the velocity of chromosome movement on the spindle falls off more rapidly than the apparent chromosome movement, measured without reference to the spindle.

In Text-fig. 12 (*b*), the velocity of chromosomes on the spindle is plotted against length of chromosome fibres (data taken from the mean curve of Text-fig. 7 (*c*)). The curve is no longer as close an approximation to a straight line as Text-fig. 12 (*a*), but shows a tendency to flatten out at either end. While it does not indicate a contractile force conforming exactly to Hooke's law over its whole range, it is still the sort of curve that might be expected from a biological contractile mechanism. (Cf. the force-extension curve for muscle, Ramsey & Street, 1940.) It is interesting to note that Östergren (1945) as a result of his work on the equilibrium position in the metaphase plate of *Anthoxanthum* trivalents, also concludes that there is a contractile force at work, proportional to the distance between centromere and pole.

Additional evidence for the contractile hypothesis is afforded by several features of anaphase seen in our phase-contrast records. The first of these concerns the form of the daughter chromosome groups in early anaphase. These often assume a bow shape, with the concave surfaces facing each other. This can be seen in Text-fig. 11 where spindle and chromosome outlines are traced from a biframe record of anaphase. The metaphase plate is of greater diameter than that of the spindle; and those chromosomes which extend beyond the visibly birefringent region of the spindle lag behind the others in early anaphase. This suggests that the spindle at its circumference is not sufficiently orientated to show up in polarized light, and that this zone is less effective in moving its appropriate chromosomes than is the better-developed central region.

A second point concerns the 'asymmetrical jerk', which is occasionally seen in early anaphase, where the forces moving the chromosomes are temporarily so far out of balance on each side, that one chromosome group is dragged the wrong way for a brief period. A difference in velocity of each group of daughter chromosomes could be interpreted on the repulsion hypothesis by assuming a difference of viscosity in the two halves of the spindle; but the actual reversal of motion can only be regarded as due to contraction, temporarily out of balance on each side.

In those instances of the asymmetrical jerk so far seen, no record of the spindle changes exists and it is therefore doubtful whether the spindle shares in the jerk or not. If this were so, the unbalanced contraction would have originated in the postulated astral contractile mechanism beyond the spindle (see later). We think, however, that an asymmetrical contraction of the spindle fibres is the more probable explanation.

Evidence from polarized light observations

The typical metaphase spindle is somewhat brighter towards either pole, while shading off rapidly at the edges. There is every reason to suppose that the spindle is symmetrical in both dimensions about its long axis, so that its depth (perpendicular to the plane of the photograph) is equal to its width. It is therefore surprising that the birefringence of the spindle is greatest at the poles, where the cross-section is least. This fact can be interpreted in two ways: either the density, or the degree of orientation of birefringent material must there be greatest.

Polarized light gives no direct means of deciding between these alternatives, or of estimating their relative importances. But if there is a greater density of protein matter near the poles, as, for instance, a number of discrete continuous spindle fibres would imply, we should expect the refractive index to be higher there than elsewhere. Phase-contrast microscopy, which is sensitive to refractive index differences, gives no indication that this is so. At present, therefore, we incline to the view that the centrosomes are the centres of orienting forces rather than regions of higher protein density, and that the spindle and asters are the manifestation of the orientations produced by these forces. If this interpretation is correct, then by analogy with muscle birefringence, the spindle must be fairly completely oriented near the poles, and less well oriented, by a factor of at least 5, at the equator.* Of the nature of the orienting forces that may be at work in the spindle and asters, we can so far say nothing.

If this interpretation of spindle structure is correct, then it seems logical to regard the centrosomes and the orienting force they exert, as the agency through which a co-ordinated contractile mechanism is built up from isotropic protoplasm. Our observations throw no light as yet, on the molecular nature of this contractile mechanism.

It is a general feature of contractile mechanisms (Schmidt, 1937), that their birefringence drops as they shorten. Schmidt (1939) observed a decrease in spindle birefringence in the sea urchin, and used it as an argument for a contractile mechanism. We have noticed such a drop during anaphase in the chick, although the spindle does not become completely isotropic. We are inclined to think that this is because the available contractile material is not all associated with the chromosomes, but forms a continuous structure with material from the opposite pole. This residual birefringence in the spindle presumably corresponds to the continuous spindle fibres of various authors. (See Schrader, 1944.)

The process of spindle lengthening can be interpreted in two ways. We may suppose that the centrosome, and a mass of nearby material moves bodily through the cell, while fresh material pours in somewhere at the centre. Alternatively, we must suppose that the centrosome alone moves, creating fresh spindle in its wake so that the molecules that constituted the pole at one moment, are some way into the spindle at the next. At present we see no mechanism to account for the second alternative, while an obvious one exists for the first. For if one set of oriented micelles, namely those in the spindle, are induced to contract, it seems reasonable, indeed almost inevitable, that the other set in the asters will do the same. If the astral micelles have some form of anchorage in the cytoplasm or on the cell walls, then their contraction is likely to lead to spindle elongation. There is some evidence for the anchorage of astral rays on to cell walls, e.g. Heilbrunn (1920).

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* The coefficient of birefringence of living muscle is about 0·0025 (Fischer, 1941). A slice of muscle 1 μ thick would therefore give a path difference of $0{\cdot}0025\mu = \lambda/200$. The coefficient of birefringence of the spindle material can hardly be more than that of muscle, and may well be less. In fact, the poles of the spindle, which are only 1 μ thick or less, show a path difference of $\lambda/500$.

Finally, we may consider what light our polarized light studies of the spindle throw on the tactoid hypothesis of mitosis (Pfeiffer, 1939; Bernal, 1940). Since this hypothesis rests so far only on the analogy in shape between the mitotic spindle and a tactoid, it is important to compare their appearances in polarized light. Bernal & Fankuchen's (1941) illustrations of the tactoids of tobacco mosaic virus show them as uniformly bright, clear-cut bodies. Our spindle photographs show an increase in birefringence at the poles. Moreover, their smallest tactoids are thin and needle shaped, and become more spherical as they grow, while the exact reverse is true of the spindle (see Pl. 2, fig. 2).

The case for the contractile mechanism discussed above is based on a 'traction fibre' hypothesis of mitosis, the general evidence for which has recently been ably summarized by Cornman (1944). Following general custom, we have used the term 'spindle fibre'. Difficult though it is to suggest an alternative, the term 'fibre' is misleading, for it suggests a discrete and continuous structure, near, or within the limits of microscopical visibility. We should rather think in terms of a much lower order of dimension of aggregations, perhaps no larger than the myosin micelle, of diameter about 50 A. Such small molecular aggregates, or micelles, are likely to be quite flexible, and to anastomose between themselves, thus forming a meshwork capable of varying degrees of orientation. Even a structure with a relatively low degree of orientation will tend to split along the line of preferred orientation on dehydration, etc. and will thus give rise to fibres in fixed preparations. Such an oriented mass of elongated molecules will, of course, have some rigidity as Chambers (1924) found for the grasshopper spindle, and will be capable of contraction. Picken (1940) gives a good account of the properties of biological chain molecules such as we are here considering.

Finally, it remains to relate the anaphase movements of the chick to those in other types of cell. In a recent paper, Ris (1943) distinguishes four main types:

(1) The spindle remains constant in length during chromosomal migration.—*Tradescantia* staminal hair cells (Ris' conclusion from Bělar's photographs (1929)).

(2) Chromosome movement falls into two parts with an interval of rest between. In the first, the spindle remains constant in length, while the chromosomal fibres shorten, in the second the spindle elongates.—Secondary spermatocytes and embryonic cells of the aphid *Tamalia*, etc.

(3) Anaphase movement is entirely due to spindle elongation. The chromosomal fibres are very short at metaphase (under 3μ) and do not shorten further.—Primary spermatocytes of *Tamalia*.

(4) The distance between centrosomes and daughter chromosomes widens simultaneously, though not necessarily at the same rate.—Grasshopper spermato-

cytes. According to Ris, 'the spindle begins to elongate before the shortening of the chromosomal fibres is complete'. Embryonic chick cells fall into this last group.

Although it is obviously impossible at this stage to develop our suggestions in relation to other types of cell, we think it likely that variation in the relative degree of development of spindles and asters and hence of the contractile force exerted, may account for some of the differences.

SUMMARY

1. The anaphase movements of living embryonic cells of the chick in tissue culture have been studied in phase contrast illumination, and in polarized light. Ciné-photographic records of chromosome movements and spindle changes have thereby been obtained simultaneously.

2. Chromosome separation in the chick is made up of two components—a movement of chromosomes towards the spindle poles, and an elongation of the spindle as a whole.

3. The assumption is made that the force acting on a chromosome is directly proportional to the velocity with which the chromosome moves. If this assumption is accepted as an approximation, it is possible to interpret the relationship of chromosome velocity to chromosome separation and spindle-fibre length. This leads to the conclusion that anaphase movement is due, not to repulsion between chromosomes, but to a contractile mechanism operating from the spindle poles.

4. Polarized light studies indicate that the orientation of the spindle material is high at the poles, and weaker at the equator. There is a small aster at either end of the spindle.

5. The centrosomes are interpreted as centres of an orienting force, which build up the contractile mechanism of the spindle from isotropic protoplasm. It is suggested that asters may also be contractile, and cause elongation of the spindle.

6. The inadequacy of the old conception of spindle fibres is discussed in the light of more modern views on molecular behaviour.

Our thanks are due, in addition to those we have already mentioned, to many of our friends and colleagues. We are indebted to Prof. J. Gray for his interest and encouragement, and for suggesting to one of us (M. M. S.) this field of research; to Dr L. E. R. Picken, Dr M. G. M. Pryor, and the late Dr D. E. Lea for information and advice; and to the Research section of Messrs Kodak for technical information. Many of the expenses of the research were defrayed by a grant from the Royal Society to the Strangeways Research Laboratory.

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EXPLANATION OF PLATES

PLATE 2

Fig. 1. Osteoblast in division; phase-contrast illumination; × 500; photographs taken at the intervals in minutes indicated. The series begins in prophase. Notice the irregular movement of the metaphase plate, 1–8 min. Anaphase begins at 9 min., and cleavage at 11 min.

Fig. 2. Osteoblast, showing the origin of the spindle; polarized light; × 250; intervals in seconds indicated.

Fig. 3. Metaphase of a primary spermatocyte in a locust; phase-contrast illumination; × 640. Photographed at two levels, showing separate resolution of detail at each.

Fig. 4. Osteoblast, showing spindle and aster; polarized light; × 500. Spindle compensated black on white. The aster is evident at the upper end of the spindle, with the transverse rays showing white.

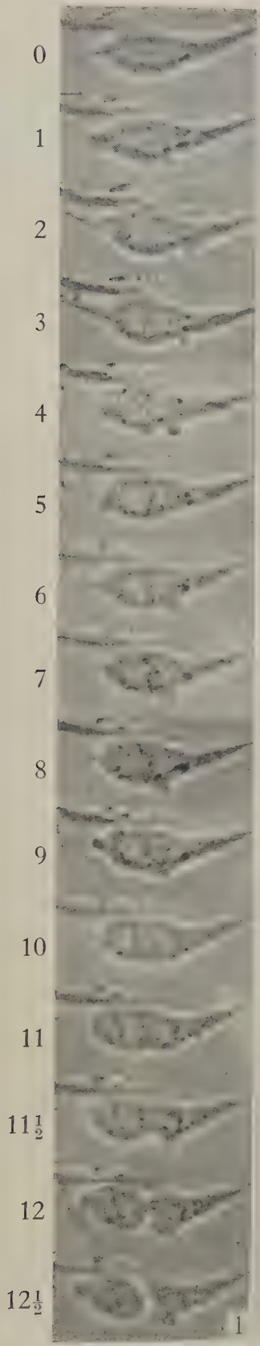
Fig. 5. Sea urchin egg, two-celled stage, showing spindles and asters; polarized light; × 350. Spindle compensated black on white. The transverse rays of the asters show white.

Fig. 6. Field of osteoblasts; polarized light; × 500. Birefringence, of same sign in every case, can be seen in spindle, cell processes, cell walls, and faintly in general cytoplasm.

PLATE 3

Fig. 1. A biframe record of osteoblasts in division; left-hand phase contrast, right-hand polarized light; × 500; intervals in minutes indicated. Spindle compensated white on black. Anaphase begins at 1 min., cleavage at 3 min. Notice the birefringence (dark) of the constricting collar at 4 and 5 min. and of the interzonal strand (light) at 6–9 min. At 9 min., the transverse band (light) referred to in the text, is visible.

Fig. 2. A biframe record of an osteoblast in division; left-hand phase contrast, right-hand polarized light; × 500; intervals in minutes indicated. Spindle compensated black on white. Anaphase begins at 1 min., and cleavage at 5 min. Notice the 'negative patch' on the right of the spindle, visible throughout, and the aster on the left, clearest at 4 min. The analysis of this record is given in Text-fig. 6.



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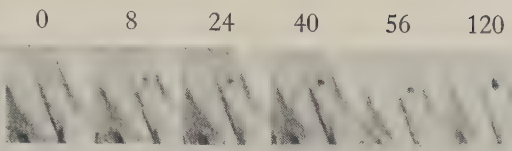

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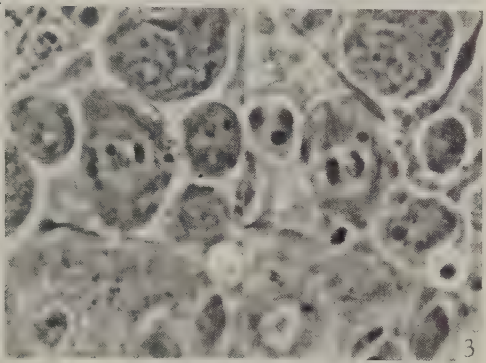

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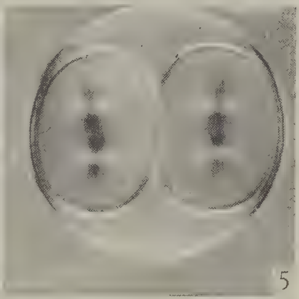

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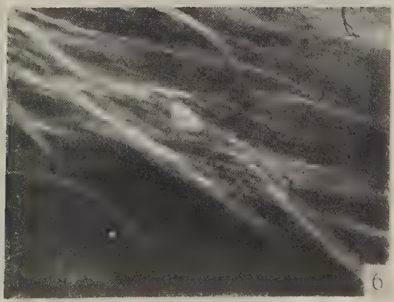

6

HUGHES AND SWANN—ANAPHASE MOVEMENTS IN THE LIVING CELL

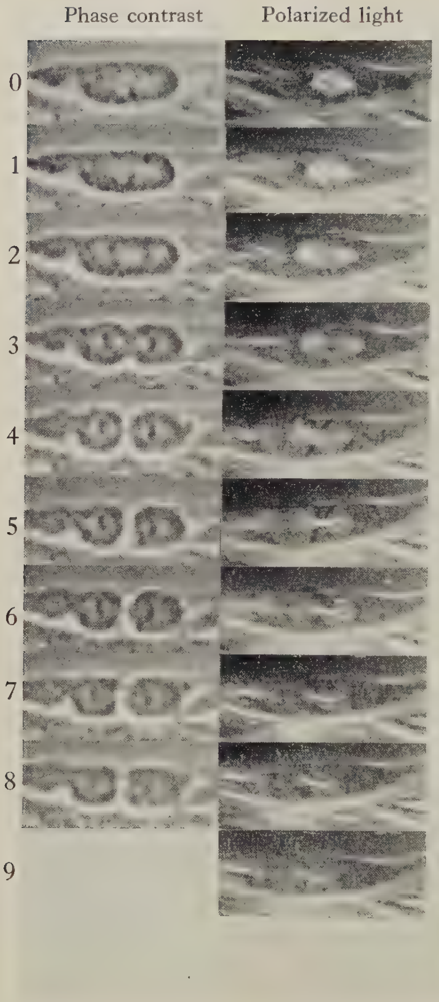

Fig. 1

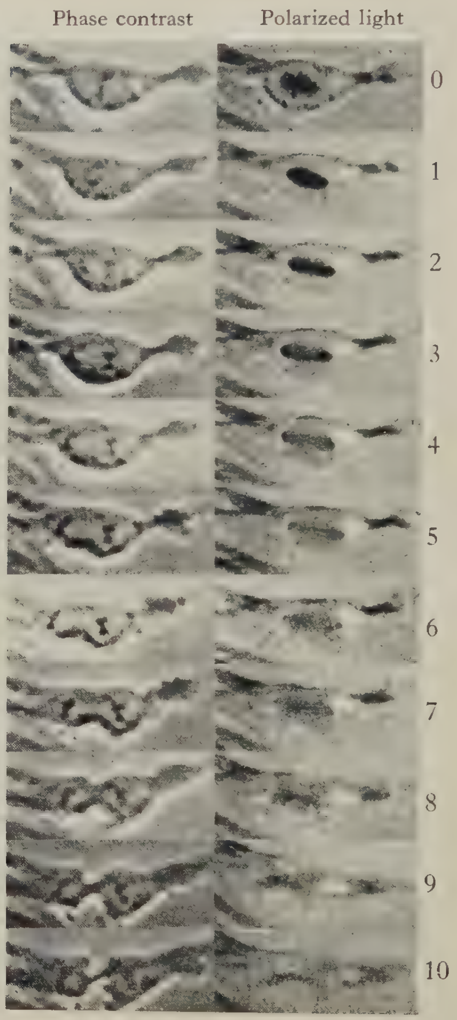

Fig. 2

HUGHES AND SWANN—ANAPHASE MOVEMENTS IN THE LIVING CELL

LABORATORY STUDIES ON THE EGG OF THE BLOWFLY *LUCILIA SERICATA* (MG.)

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(With Three Text-figures)

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INTRODUCTION

The laboratory studies of Evans (1934) on the humidity and temperature relations of *Lucilia sericata* (Mg.) eggs showed that they were ill adapted to survive at low humidities. Yet, Davies & Hobson (1935) and Macleod (1940) have since demonstrated that low humidities are of general occurrence in sheep fleeces where *L. sericata* eggs may be laid. Further, Davies & Hobson re-emphasized Evans's conclusion and expressed the opinion that a steady humidity of over 90 % R.H. for 14 hr. was necessary to ensure the hatching of eggs and the establishment of myiasis. In none of these investigations was the effect of humidity fluctuations studied. The present investigation was therefore undertaken to determine the effects on egg development and hatching of humidity fluctuations of a kind likely to occur under natural conditions. A description of humidity conditions in the fleece is being published separately (Davies, 1948). Further, a study of the egg-shell has been made, on the lines of the work of Beament (1946*a*, *b*), in order to determine the nature of the waterproofing mechanism of the *L. sericata* egg.

I. GENERAL MORPHOLOGY OF THE EGG-SHELL

(i) *Microscopic structure*

Methods. The shell of the ovarian egg about 2 days before laying was studied, when, as far as could be seen in sections, it had reached its final size and shape. At this stage the follicle cells had undergone almost complete necrosis and could no longer add material to the shell. The chorion had the same external morphological features, such as the hexagonal imprints of the follicle cells and the longitudinal hatching pleats (see Sikes & Wigglesworth, 1931) as are found in the chorion of the laid egg. Eggs were removed from flies, fixed in alcoholic Bouin, and paraffin sections made. Evidence on the nature of the shell components at this stage was compared with that on the shell of laid eggs, which were not fixed but embedded in paraffin after preliminary dehydration and clearing.

Chorion. The chorion of ovarian eggs with completely developed shells seemed identical in microscopic appearance and staining properties with that of laid eggs. About 5·0 μ thick over the main part of the shell, it was thickened to about 10·0 μ in the region of the hatching pleats and at the edges of the circular area surrounding the external micropyle (Weismann, 1863). Staining and other chemical tests showed that the chorion was composed of two main layers with a row of dark bodies forming parts of the boundary between them (Fig. 1). The outer layer, about 3·0 μ thick, was readily stained, whilst the inner layer, less readily stained, was 2·0 μ thick over most of the shell. Where the chorion was thickened, e.g. at the edges of the circular micropylar area, most of the increase in thickness was due to an expansion of this inner layer, the outer layer remaining the same thickness over all parts of the shell.

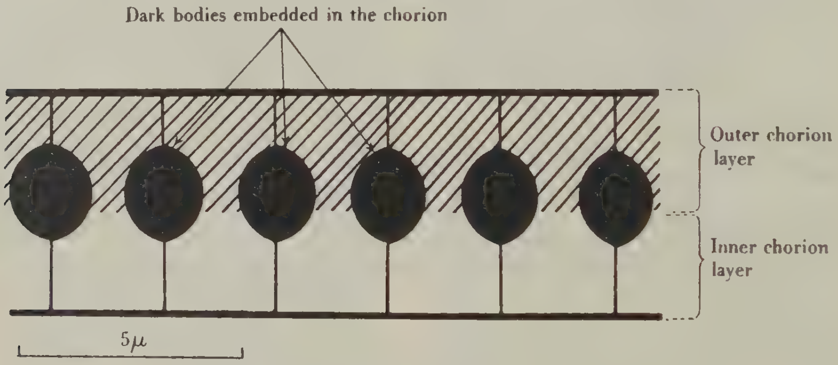

Fig. 1.

The columnar structure of the chorion of *L. sericata* eggs (Fig. 1) agreed with the description given for the eggs of other Tachinids by Pantel (1913).

The outer chorion layer was at all stages weakly stained by acid fuchsin, pale brown by Ehrlich's haematoxylin, pale blue after Mallory's triple staining, and pale green after light green, whilst the inner chorion layer was not affected by these stains. Both layers, however, stained deeply in basic fuchsin, gave a positive xanthoproteic test, and negative results with both ninhydrin and warm Millon's reagent. It was evident therefore that both chorion layers contained protein, but differences in their staining properties indicated that they were not of identical composition.

The chorion as a whole was resistant to cold concentrated nitric and hydrochloric acids, but dissolved rapidly in saturated caustic potash solution at 150° C., and more slowly in warm concentrated nitric acid.

When sections of shells were immersed overnight at room temperature in a saturated solution of *p*-benzoquinone, the outer chorion layer and the row of dark bodies (Fig. 1) became tanned to a deep brown colour over the whole of the egg-shell, whilst the inner layer remained colourless. This result was obtained both with ovarian eggs and with laid eggs. It was concluded therefore that the protein of the inner layer was already tanned—or, alternatively, so modified that no tanning by *p*-benzoquinone could occur—and that the outer chorion layer was largely composed of protein normally susceptible to tanning agents.

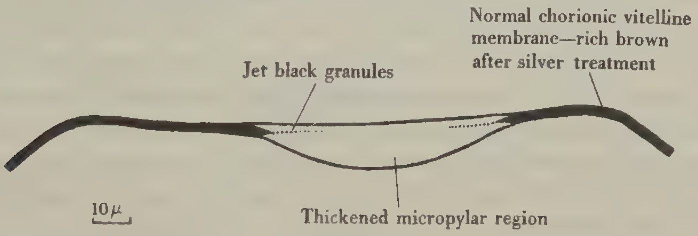
Normal chorionic vitelline membrane—rich brown after silver treatment
Jet black granules
10μ
Thickened micropylar region

Fig. 2.

The two-layered nature of the chorion, with one layer untanned and the other possibly tanned may explain some of its peculiar mechanical properties. A strip of chorion removed from an egg and mounted so that one end is attached and the other free, underwent rapid curling movements when subjected to humidity changes. When a wet needle was held near it, the chorion curled downwards. When a warm needle was held near, it curled in the opposite direction, instantly returning to its former condition when the warm needle was removed. These observations could be explained if the untanned protein of the outer chorion layer could undergo volume changes dependent on variations in air humidity, whilst the inner layer, being tanned, would be more rigid. The mechanical properties of the chorion not only affect the hatching process, but may account for various features of the rates of water loss from eggs (see p. 83).

Chorionic vitelline membrane. This membrane (hereinafter referred to as the c.v. membrane) was about 3·0 μ thick over the general surface of the egg, but thickened to about 10·0 μ in the region of the micropyle (see Pantel, 1913). It stained heavily in Ehrlich's haematoxylin, pale blue after Mallory's triple staining, green after light green, and gave a positive xanthoproteic test. It was tanned a dark brown colour in *p*-benzoquinone at room temperature overnight and dissolved rapidly in saturated caustic potash solution at 150° C. It resisted attack by concentrated nitric acid in the cold for several minutes, while on heating it dissolved more rapidly and gave off oily droplets. In sections of fixed ovarian eggs, treated with 1 % ammoniacal silver nitrate, the c.v. membrane gave the characteristic polyphenol reaction (Lison, 1936), except in the thickened micropylar region. At the edges of the non-stained micropylar region there appeared a row of very fine, jet black granules embedded in the membrane as though emerging from the thin part, but not continuous across the thick part (Fig. 2). This layer of silver-reducing granules may have been continued along the thin region of the c.v. membrane, but were invisible as such even under a 1/12 objective, possibly owing to the excessively fine dimensions of the membrane itself. In the laid *L. sericata* egg, the c.v. membrane showed no silver-reducing properties.

(ii) *Lipoid content of the egg-shell*

Ovarian egg. Eggs removed from the ovary some 2 days before they were due to be laid had no resistance to desiccation, although the shell as seen in sections had reached its final dimensions and the follicle cells had undergone necrosis. Such eggs collapsed immediately in saturated sodium chloride solution at 19° C., and swelled and burst in distilled water, indicating that the shell was freely permeable to water in both directions. Fragments of chorion and c.v. membrane from these ovarian eggs, when placed in cold concentrated nitric acid, either alone or saturated with potassium chlorate, showed no change within the first few minutes, but rapidly dissolved in both these media on heating. No oily droplets were formed during their disintegration. The shell of the ovarian egg does not therefore seem to contain any appreciable lipoid material.

Laid egg. The shell had the opaque white appearance of the typical Muscid egg. The chorion, considering its thin nature (5·0 μ thick) was very strong. The water permeability of the shell was now much lower. Most eggs could complete development whilst immersed in saturated sodium chloride solution or concentrated picric acid at 19° C. On placing pieces of chorion or c.v. membrane in cold concentrated nitric acid or cold Schulze's medium, oily droplets were slowly given off. On heating, droplets were rapidly produced, and these could be stained by Sudan III. This result is in marked contrast to that obtained with the same membranes from ovarian eggs. It appears therefore that both chorion and c.v. membrane of laid eggs contain appreciable lipoid material, whilst in ovarian eggs they contain little or no such substances, although at the latter stage the follicle cells have completed their activity and already undergone necrosis. No layers in the shell of laid eggs were selectively stained by either Sudan III or Sudan Black B. Treatment of the chorion and c.v. membrane in chloroform or ether for 12 hr. at 30° C. did not visibly affect the bulk of their lipoid content. After this treatment they gave Sudan staining droplets in warm nitric acid in quantities comparable to that given off by untreated shells. It appears, therefore, that lipoid contained in the chorion and c.v. membrane of the laid egg is bound and cannot be removed by fat solvents. The relatively large quantities of oily droplets given off by the chorion in nitric acid suggests that both its layers contain lipoid incorporated into the protein structure. Moreover, this lipoid would appear to be a product of the öocyte, because it does not appear in the shell until the follicle cells have completely degenerated. Since most of the lipoid cannot be removed by fat solvents it probably forms a lipo-protein with the protein of the shell already secreted by the follicle cells. With the incorporation of this lipoid into the chorion and c.v. membrane, there occurs a marked increase in the rigidity and mechanical strength of the egg-shell. It is to be expected that lipidization of the protein of the shell would increase its rigidity. On the other hand, this process alone would not be expected to reduce markedly its water permeability. As will be shown later the appearance of bound lipoid in the shell is not itself responsible for waterproofing the egg.

The effects of fat solvents, such as ether, chloroform and carbon tetrachloride on the water permeability of intact laid eggs was gauged by comparing their behaviour in saturated sodium chloride solution at 19° C., before and after treatment in a particular fat solvent. It was found that immersion in chloroform at 30° C. for 12 hr. produced a radical increase in the permeability of the shell, and made it freely permeable to water in both directions. This indicated that chloroform soluble material responsible for waterproofing the egg-shell was removed or disorganized during the treatment.

When batches of eggs, each batch laid by one fly, were immersed within 15 min. of laying in saturated saline at 19° C., it was found that a small proportion (usually about 10 %) of the eggs in each batch showed signs of water loss by shrinkage within 15 min. The following observations suggested that these non-waterproofed eggs were those that were laid last by each fly. Eggs were dissected from flies in process of ovipositing, and their behaviour in saturated saline at 19° C. compared with that of eggs already laid a few minutes earlier by the same flies. About half the eggs, due to be laid within the next few minutes from various positions in the oviducts and vagina, showed signs of water loss after 15 min. under the above conditions—the rest were unaffected. Only 5 % of the eggs already laid by the same flies were of this non-waterproof type. The process of waterproofing the shell would thus appear to occur shortly before laying, and to be unfinished in some eggs when they are laid.

The following observations show that the waterproofing of the egg was not due to the bound lipoid which appeared in the shell shortly before laying. Eggs of the non-waterproofed type, macroscopically identical with laid eggs, were removed from the oviducts of a fly in process of ovipositing. Fragments of chorion and c.v. membrane from such eggs were placed in warm concentrated nitric acid, and droplets stained by Sudan III were given off. This showed that although the shell protein had been lipidized at this stage, the eggs were still permeable to water in both directions. The waterproofing of the egg must therefore be due to some other change in the shell occurring shortly before laying.

Occasionally laboratory culture flies laid complete batches of non-waterproof eggs with slightly transparent chorions. Such eggs shrivelled up at all humidities below saturation and did not complete development. Gough (1946) records that the wheat bulb fly (*Leptohylemyia coarctata* Fall) laid a few eggs of this type when the egg batches in question contained more eggs than the average number of ovarioles in that species. In *L. sericata*, eggs which shrivelled up were not observed to be more frequent in large batches than in small ones.

(iii) *Effect of temperature on rate of water-loss*

Batches of eggs, weighing 20–30 mg. freshly laid by a laboratory culture of flies, were teased apart on fragments of silver foil so that they formed a layer one egg thick and examined under a binocular microscope to ensure that damaged eggs were not used in experiments. After storage in dry air at room temperature for 30 min. to remove all water from the outside of the shells they were re-examined. The rates of water loss from such batches when exposed to dry air at various temperatures were then measured. The foil with eggs attached was suspended in a corked flask fitted with a thermometer and containing phosphorus pentoxide as desiccant, the eggs being a standard distance above the drying agent. This apparatus was similar to that employed by Wigglesworth (1945). The flask was placed in a thermostatically controlled oven. Weighings were made by a torsion balance (50 mg./0·05 mg.). Owing to the large surface area, water loss decreased rapidly with time at high temperatures owing to depletion of water in the eggs. Short exposures of only 15 min. at each high temperature were therefore used, longer exposures of $\frac{3}{4}$ or 1 hr. being employed at lower temperatures. Surface areas were estimated by making camera lucida drawings of eggs squashed flat under a cover-slip. In this way the water loss was expressed as mg./sq.cm. surface/hr., and this provided data comparable to that obtained by Wigglesworth (1945) on transpiration through the insect cuticle and by Beament (1946*a*) on that through the shell of the *Rhodnius* egg.

The temperature/water-loss curve obtained in this way is shown in Fig. 3. It will be seen that the rates of water loss at temperatures of 20–35° C. were low and increased very slowly with rise in temperature within this range. But at about 38° C. the water permeability of the shell increased abruptly. It will be obvious from Fig. 3 that the rapid water loss at the higher temperatures necessitated the use of separate egg batches for each new temperature. This curve (Fig. 3) is very similar to that obtained by Beament (1946*a*) in comparable experiments on the *Rhodnius* egg, which he showed was waterproofed by a wax layer. The curve is also similar to those obtained by Wigglesworth (1945) in experiments on the water loss from adult, pupal and larval insects of various orders, and by Beament (1945) for the permeability of membranes covered with films of waxes extracted from insect cuticles. It appears therefore that the *Lucilia sericata* egg is waterproofed by a lipoid layer rather similar to that of the *Rhodnius* egg and to the waterproofing layer of insect cuticle. This lipoid waterproofing layer has a 'critical temperature' in the region of 38° C.

Other features in the effects of temperature on the permeability of the *Lucilia sericata* egg-shell are similar to those for insect cuticle. The rate of water loss in dry air at 30° C. was found to be of the order of 0·5 mg./sq.cm./hr. (Fig. 3). Batches exposed to 50° C. and then placed in dry air at 30° C. lost water at slightly over 1·0 mg./sq.cm./hr. Exposure to 40° C. for 30 min. had little or no effect on the subsequent water loss rate in dry air at 30° C., whilst 10 min. at 50° C. produced the increase noted above. High temperatures appear to cause a permanent increase in the permeability of the shell. This was found to be the case with insect cuticle in similar experiments on *Rhodnius* nymphs by Wigglesworth (1945). Exposure to 50° C. greatly increased the permeability of the *Lucilia sericata* egg-shell in both directions. Batches of eggs desiccated at 50° C. until they were heavily dimpled regained their water slowly in saturated air at 37° C., and more rapidly in saturated air at 50° C., so that the dimples in the shells disappeared. For example, a batch of eggs weighing 20·15 mg. was desiccated in dry air at 50° C. for 40 min. Its weight was then 9·8 mg. Placed in saturated air at 37° C. it regained 1·65 mg. in 3·5 hr.—a rate of approximately 0·47 mg./hr. It was then placed in saturated air at 50° C. and regained 2·75 mg. in 1·3 hr.—a rate of approximately 2·1 mg./hr. In addition these eggs eventually regained water to such an extent that they weighed about 5 % more than their original weight, prior to initial desiccation, so that they appeared slightly fatter than normal laid eggs. Evans (1934) showed that eggs, partly desiccated at temperatures below the critical temperature of 38° C. found in the present work, did not regain water in saturated air.

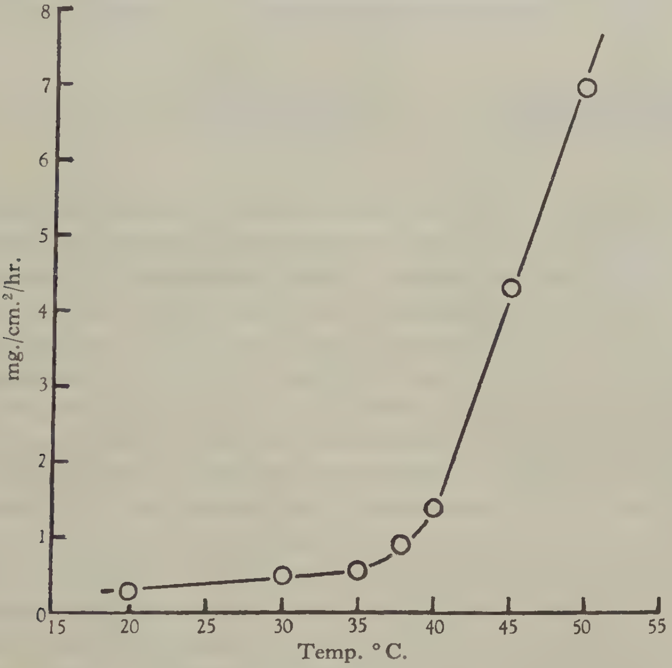

Fig. 3. Temperature/water loss curve, *Lucilia sericata* egg.

The temperature/water loss curve (Fig. 3) was found to be similar in live and in dead eggs killed with ammonia fumes 24 hr. before use. Thus the waterproofing of the egg is not due to an active physiological mechanism.

(iv) *Effect of a detergent and abrasion on shell permeability*

The I.C.I. detergent C09993 was used (see Wigglesworth, 1945). Applied as a watery paste to the outer surface of the intact c.v. membrane after the chorion had been stripped off, it caused a marked increase in the rate of water loss through this membrane in dry air at 19° C. Eggs so treated collapsed completely in 12 min. Untreated controls with chorions removed, collapsed under similar conditions in 1 hr. This suggests that an emulsifiable material was present on the outside of the c.v. membrane and was responsible for reducing its permeability to water. Application of the detergent to the outer surface of the chorion of the intact egg also affected the rate of collapse of eggs in dry air (Table 1).

Table 1. *Effect of* C09993 *applied to outside of chorion, twenty eggs in each group*

	Time taken to collapse in dry air 19° C.
Untreated	Not collapsed within 18 hr.
Whole egg-shell treated	Completely collapsed in 15 hr.
Wide end of egg treated	Shell dimpled but not collapsed in 18 hr.
Micropyle end of egg treated	Completely collapsed in 15 hr.

These results (Table 1) suggest that the detergent penetrated the chorionic micropyle and, spreading on to the c.v. membrane, affected its water permeability. Eggs minus the chorion, when placed in strong saline showed slight signs of water loss within 30 min.; after washing in ether for 5 min. they collapsed completely in 1–2 min. in strong saline. On return to distilled water they regained their original shape within 10 min. and occasionally took up water to such an extent that they burst. Freshly laid eggs minus the chorion required only 5 min. washing with ether to become as permeable as described above. Similar eggs, killed in ammonia fumes and stored for 2 days required immersion for 30 min. to produce the same effect. These results again suggest the existence of a lipoid layer on the outer surface of the c.v. membrane.

The effects of abrasion on the permeability of the shell was gauged by comparing the dimpling rate of eggs drawn several times through a layer of alumina dust on a glass slide, with those of untreated controls. It was found that abrasion of the outside of the chorion produced no appreciable increase in the rate of water loss in dry air at room temperature. Eggs with chorions removed, drawn through alumina so that the outside of the c.v. membrane was exposed to abrasion, lost water very rapidly. Such eggs became heavily dimpled within 3 min. and completely collapsed in 10 min. by which time untreated control eggs with chorions removed had not yet lost their original fat sausage-shape. When the c.v. membrane of eggs with chorions removed was dusted, but abrasion of the membrane avoided by keeping them stationary and not moving them through the dust, no marked increase in water loss occurred. This suggests that the waterproofing layer was not a mobile oil which could be absorbed by alumina dust.

All these experiments strongly suggest that the waterproofing layer is situated between the c.v. membrane and the closely fitting chorion. When the chorion is removed damage to the waterproofing layer would be expected, so that the egg minus the chorion would become very susceptible to desiccation. Possibly some of the waterproofing lipoid is partially removed as a coating on the inside of the chorion. The statement of Evans (1934) that the chorion is responsible for the resistance of the *L. sericata* egg to desiccation is therefore misleading.

(v) *Site of water loss*

Using the rate of dimpling of the shell in dry air as a measure of water loss, occlusion of the external micropyle and/or the longitudinal hatching strip of the chorion with a layer of cellulose paint or paraffin did not affect the rate of water loss from eggs at room temperatures. Eggs so treated completed development at appropriate humidities. It seems that, in effect, water loss occurred over the whole surface of the shell, and not through a restricted area such as the micropyle or hatching strip.

II. EFFECTS OF EXPOSURE TO DIFFERENT HUMIDITIES

(i) *Methods*

Eggs were obtained from a laboratory culture of flies kept at 22–25° C. By careful observation, after meat was placed in the cage, it was possible to determine the time of laying to within 5–10 min. Experiments were made at 30, 34, 37, 38 and 39° C. in electric incubators giving fluctuations of no more than ±0·5° C. from these temperatures. Relative humidities of from 0 to 95 % were maintained in desiccators by means of sulphuric acid solutions, made up at 25° C. according to the data of Wilson (1921). The specific gravity of the acid was checked at intervals by weighing 10 c.c. samples, and the humidity within the desiccators checked by paper hygrometers or cobalt chloride papers (Solomon, 1945). Eggs, either separated or as complete batches, were incubated within the desiccators, on 3 × 1 in. slides, which could be transferred from one desiccator to another during the incubation period. This involved little disturbance in humidity conditions. In this way, the effect of short periods at very low R.H.'s could be measured. Where time of hatching (or variations in time of hatching within a group of eggs) had to be determined, desiccators fitted with flat plate glass lids were used to facilitate observation of hatching. In all experiments a set of controls was allowed to develop and hatch at 100 % R.H.

To provide data comparable to that obtained by Larsen (1943) on the eggs of dung-breeding Diptera, experiments were also carried out in vessels similar to those used by her. These were solid watch-glasses sealed by a glass plate, on the underside of which the eggs in question were attached (see Larsen 1943, fig. 2). In the present experiments humidity in these vessels was controlled by sulphuric acid/water mixtures placed in the watch-glasses, so that the eggs lay within 1 cm. of the surface of the desiccant.

Eggs laid by flies from 1 to 4 weeks old were used. No significant differences were found in their humidity relations which could be attributed to differences in age of flies.

(ii) *Minimum humidity for development*

The minimum humidity for development at 37° C. was 50 % R.H. (23·53 mm. sat. def.). The proportion of eggs reaching the pre-hatching stage at this humidity varied considerably between different batches laid by separate flies, as shown in the results of fourteen experiments given below:

% eggs completing development at 50 % R.H. 37° C.	92	40	42	66	50	80	11
	6	16	20	29	18	45	16

Some of these results were obtained by incubating simultaneously in the same humidity vessel eggs laid by different flies. Even in such circumstances the proportion of eggs completing development showed differences between batches although they had been treated identically. There must, therefore, have been considerable variation in the resistance to desiccation of eggs from different flies, as well as variation within a single batch laid by the same female.

The results of experiments in solid watch-glasses as used by Larsen (1943) were substantially similar to those in which desiccators were used as humidity chambers.

To confirm the above results, eggs were obtained from wild flies in the field by attracting them to oviposit on sheep, using the technique of Hobson (1937). Results identical with those described above were obtained with such eggs. Thus the low minimum humidity figure of 23·5 mm. sat. def. found in the present work could not be due to using laboratory flies accidentally selected for desiccation-resistant eggs. In the circumstances it is, therefore, impossible to account for the much lower figure of approximately 17 mm. sat. def. obtained by Evans (1934).

In saturated air at 37° C. development was found to take 7·6–7·8 hr., a figure agreeing closely with that given by Wardle (1930). At 50 % R.H. at the same temperature, development was found to take 11·75–14·0 hr. thus showing considerable retardation in the rate of development. Retardation of development in *L. sericata* eggs, caused by low humidities, was also found by Evans (1934) and in the eggs of dung-breeding Diptera by Larsen (1943).

At 34° C. the minimum humidity for development was found to be about 40 % R.H. (23·84 mm. sat. def.), when 70 % of the eggs completed development, and at 30° C., 25 % R.H. (23·86 mm.), when 68 % of the eggs completed development. The remainder of the eggs died at an early stage in development. The percentages given above again showed great variation from one experiment to another.

Table 2

Humidity % R.H.	Percentage eggs completing development	
	38° C.	39° C.
95	90	75
80	95	10
75	95	0
70	0	0
65	0	0

Evidence has been given in this paper that the waterproofing layer of the *L. sericata* egg has a 'critical temperature' in the region of 38° C. The humidities required by eggs to complete development at 38 and 39° C. were therefore investigated. The results of one experiment are shown in Table 2. It will be seen that at 38° C. the minimum humidity for development of eggs was about 75 % R.H., and at 39° C. about 80 % R.H. Since, at these temperatures and at 95 % R.H., 90 and 75 % of the eggs respectively completed development it is concluded that the high temperatures alone were causing some mortality apart from the effects of humidity. (At 38 and 39° C., fewer eggs completed development in 100 than at 95 % R.H. This was presumably due to condensation on the outsides of the eggs interfering with respiratory exchange. A similar phenomenon is mentioned by Larsen (1943).) At 38° C. development was retarded by high temperature alone, but this itself does not account for the much higher humidity required for development, since at 80 % R.H. 38° C. the product of saturation deficiency × developmental time (hr.) for *L. sericata* eggs is about 136, whilst at 50 % R.H. 37° C. it is about 282. Since at 95 % R.H. quite high proportions of eggs completed development at 38 and 39° C., this sharp rise of the minimum humidity must be due in part to the harmful effects of the high temperature on the lipoid layer, leading to increased water-permeability of the shell.

In view of the fact that the waterproofing layer of the egg has a 'critical temperature' in the region of 38° C., the maximum temperatures found in their natural environment—the sheep's fleece—is of interest. Burtt (1945) found that the temperature as measured by a thermocouple on the face and ears of a sheep, was 35–37° C. In the present work temperature readings were taken by inserting a clinical thermometer into the fleeces of two sheep under summer field conditions. Sixteen readings taken against the skin at the midback averaged 38·6° C. when the sheep were in the shade. When their fleeces were warmed by direct sunlight the skin temperature rose to 39·8° C. (average of three readings). Temperatures at 3 cm. off the skin, within the thickness of their fleeces varied greatly, frequently being below 30° C. when the sun was clouded, but rising above 40° C. in direct sunlight. These observations indicate that *L. sericata* eggs laid very close to the skin of the sheep may have to develop at temperatures approaching the 'critical temperature' of their waterproofing layer.

(iii) *Minimum humidity for hatching*

Davies & Hobson (1935) found that at 37° C. *L. sericata* eggs required 90–100 % R.H. for rapid hatching. In the present experiments at these high humidities variation in hatching in a batch was about 10 min. With progressively lower humidities, from 90 to 60 % R.H. hatching was found to be less complete, and much slower. The variation in hatching times of the various eggs in a batch was about 2 hr. at 60 % R.H. 37° C., and the final percentage of eggs which hatched varied from 40 to 90. The remainder of the eggs at these humidities all contained fully developed larvae imprisoned within the shell. Experiments supported the conclusion of Davies & Hobson (1935), that hatching was completely prevented at 50 % R.H. 37° C. At this humidity, however, some hatching occurred in large batches of eggs, incubated as laid by the flies. Forty-three complete unseparated batches were incubated at this humidity. Some hatching occurred in thirteen of them whilst still in air at 50 % R.H. 37° C., but never more than 20 % of the total eggs in each batch hatched. This hatching at 50 % R.H. was presumably due to some of the eggs being protected, by close packing, against the effects of the low humidity. In one large mass of approx. 1200 eggs laid by six flies, about 200 eggs hatched in air of 50 % R.H. 37° C. It was observed that the eggs which hatched at this low humidity were usually those on the edges of the underside of the egg batch. Eggs in the middle of the batch contained fully developed larvae, no doubt unable to break out of their egg-shells because the latter were cemented together into a solid mass by the dried covering of accessory gland secretion coating the eggs. All larvae which emerged at 50–70 % R.H. 37° C. were weak and shrivelled looking—the 'chiton-larvae' of Larsen (1943)—and died soon after hatching.

Davies & Hobson (1935) showed that eggs incubated in saturated air to within 20 min. of hatching, did not hatch if they were transferred to air of 50 % R.H. 37° C.—the fully developed larvae being imprisoned within the hardened chorion. Further experiments on this point have shown that larvae could withstand such imprisonment for about 3 hr. Up to that time, the bulk of the larvae hatched when eggs were transferred back to saturated air. Larvae imprisoned for periods longer than 3 hr. showed increasing mortalities—a 100 % mortality being reached after approximately 4 hr. imprisonment.

(iv) *Effects of variations in humidity*

Immediately after laying, eggs were incubated at 37° C. at humidities of 0, 30, 40 and 50 % R.H. for varying periods. They were then quickly transferred to 100 % R.H. 37° C. and allowed to complete development. Mortalities produced by exposures of various lengths to these low humidities are shown in Table 3.

Table 3. *Percentage mortality of eggs exposed to low humidities at 37° C. immediately after laying*

Hr. exposed	0% R.H.	30% R.H.	40% R.H.	50% R.H.
1·0	10	0	—	—
1·8	18	0	—	—
3·0	14	0	0	—
3·6	78	0	0	—
4·0	100	0	0	0
4·25	100	18	0	0
5·0	—	12	0	2
6·0	—	35	4	—
7·0	—	47	52	1
8·0	—	82	58	4
8·25	—	76	—	—
9·0	—	100	100	18
9·5	—	100	100	8
12·0	—	—	—	52
13·0	—	—	—	72
14·0	—	—	—	68
14·5	—	—	—	84
14·75	—	—	—	92
15·0	—	—	—	100

It will be seen that at 37° C., comparatively long exposures to air of 40 and 30 % R.H. are required to produce a 50 % mortality. Since the R.H. of the air at the base of the sheep's fleece in summer rarely falls below 30 % R.H. (Davies & Hobson (1935), Macleod (1940)), eggs laid in a dry fleece would therefore be expected to survive if the humidity rose rapidly during the incubation period. Consecutive readings of fleece humidity at standard points in the fleece (Davies, 1948) show that such rises in fleece humidity do occur under natural conditions.

It was found that eggs incubated immediately after laying in saturated air at 37° C. were particularly susceptible to desiccation if subsequently incubated at lower humidities. About 30 min. in saturated air was sufficient to cause this effect. Table 4 shows the results of one experiment in which eggs from one batch were incubated in saturated air at 17, 30 and 37° C. for 90 min. and then placed for the rest of their developmental period in 55 % R.H. 37° C. Twenty eggs in each group were used. It will be seen that this 'conditioning' effect was most marked at 37° C. and much less so when the period in saturated air was at 30 and 17° C. It is well known that the chorion of the Muscid egg hardens in dry air, and softens in humid air. It would appear that *L. sericata* eggs are laid with the chorion in the 'hard' condition, and that incubation in saturated air is needed to soften them. The conditioning of eggs by placing them in saturated air for a time may possibly be explained by this softening of the chorion. The 'hard' chorion may allow less rapid water loss than when it has been softened. Water loss through the cuticle of the wireworm was found by Wigglesworth (1945) to diminish rapidly at very low humidities, and he suggested that the lowered permeability resulted from the drying of the cuticle. In the present study it was noticed that eggs conditioned in saturated air at 37° C. for 90 min. became dimpled in a shorter time than unconditioned eggs, which had been kept on the bench at about 65 % R.H. for the same time, when both groups were placed in dry air. This was presumably due to the softened chorion of the 'conditioned' eggs being less resistant to dimpling than the harder chorion of the 'unconditioned' eggs. Dimpling of the shell would be expected to cause deformation of the lipoid waterproofing layer, especially at the edges of the dimple, leading to increased water loss and thus even quicker dimpling. The shells of the 'unconditioned' eggs would resist initial dimpling, and thus would lose water more slowly so that more of the eggs would complete development. Since softening of the chorion occurred readily in saturated air at 17° and 30° C. as at 37° C., the less marked 'conditioning' effect at the lower temperatures cannot be explained at present.

Table 4. *Effect of preliminary incubation in saturated air*

Treatment	Percentage eggs completing development	Percentage eggs dying at early stages
Incubated at 100% R.H. 37° C. for 90 min.; then transferred to 55% R.H. 37° C. for rest of incubation period	10	90
Incubated at 100% R.H. 30° C. for 90 min.; then transferred to 55% R.H. 37° C. for rest of incubation period	72	28
Incubated at 100% R.H. 17° C. for 90 min.; then transferred to 55% R.H. 37° C. for rest of incubation period	85	15
Whole incubation period at 55% R.H. 37° C.	100	0

In view of this 'conditioning' effect, experiments of the following type were carried out. A single large egg batch was divided in two, and each half weighed. One half (*A*) was 'conditioned' for 90 min. at 100 % R.H. 37° C., reweighed, then placed in 0 % R.H. 37° C. for 3 hr. The other half (*B*) was incubated on laying at 0 % R.H. for 3 hr. at the same temperature. In one such experiment, batch *A* in 3 hr. at 0 % R.H. 37° C., lost 38 % of its original weight whilst batch *B* lost only 27 % under the same conditions. This is in agreement with the higher mortalities recorded for 'conditioned' eggs.

DISCUSSION

The effects of fat solvents, a detergent, an abrasive dust and high temperatures, on the permeability of the *L. sericata* egg-shell leave little doubt that it is waterproofed by a lipoid layer laid down by the öocyte and situated between the chorion and the c.v. membrane. Its waterproofing mechanism thus appears to be similar in essentials to that described by Beament (1946*a*) for the egg of the hemipteron *Rhodnius prolixus*, and by Wigglesworth (1945) and Beament (1945) for the waterproofing mechanism of insect cuticle. It is interesting to note that an oily layer has been found by Christophers (1945) in the *Culex pipiens* egg, lying between its exo- and endochorion.

The present work showed that bound lipoid material appeared both in the chorion and the c.v. membrane of the *Lucilia sericata* egg shortly before laying, and that this lipoid alone did not waterproof the shell. The main function of this bound lipoid would appear to be to strengthen the shell prior to laying down the waterproofing layer. A thin waterproofing layer of orientated molecules would not be effective if laid down on a shell that was soft and easily deformed. Beament (1946*a*) showed that the waterproofing layer of the *Rhodnius* egg is not laid down until the shell is fairly rigid after the follicle cells have completed the secretion of the endochorion.

Humidity experiments described in this paper showed that *Lucilia sericata* eggs could withstand considerable desiccation at 37° C., with some eggs completing development at 50 % R.H. (23·5 mm. sat. def.). The work of Larsen (1943) showed that eggs of dung-breeding Diptera required much higher humidities for development. It has been shown by previous work (Macleod, 1940; and by Davies, 1947) that the natural environment of the *L. sericata* egg is normally much drier than that of the eggs of dung-breeding flies. The work of Beament (1946) showed that water loss through the waterproofed *Rhodnius* egg-shell in dry air at 30° C. occurred at about 0·1 mg./sq.cm./hr. (Beament, 1946*a*, fig. 2). Under the same conditions water loss through the *Lucilia sericata* egg-shell was found to be in the region of 0·5 mg./sq.cm./hr. Even after allowing for error in calculating the surface area of *L. sericata* egg batches in this work, it seems that the shell of the latter is more permeable to water than is that of *Rhodnius*. This greater permeability, coupled with much smaller size and larger surface/volume ratio, account for the more humid conditions required by *Lucilia sericata* eggs for development.

The results of experiments on the humidity relations of the *L. sericata* egg in relation to blowfly strike will be discussed in a later paper.

SUMMARY

The chorion of the *Lucilia sericata* egg is shown to be composed of two main layers, both of protein; the outer can be tanned by *p*-benzoquinone, but the inner is apparently already tanned. The chorion and the chorionic vitelline membrane are both lipidized shortly before the egg is laid. This makes both structures more rigid, but does not waterproof the shell. After the lipidization process is completed, a lipoid waterproofing layer is laid down by the öocyte, between the chorion and the chorionic vitelline membrane. This waterproofing layer has a critical temperature in the region of 38° C., and can be damaged by an abrasive dust, emulsified by the detergent I.C.I. C09993, and removed by chloroform at 30° C. in 12 hr.

The minimum humidity for development of *L. sericata* eggs at 37° C. has been found to be 50 % R.H. At 1–2° C. above this temperature eggs require about 80 % R.H. to complete development. Eggs at 37° C. can withstand fairly long periods of humidities below 50 % R.H., provided they have not been previously incubated in saturated air at that temperature. The latter treatment, even if continued for only 30 min., makes eggs far more susceptible to desiccation when subsequently incubated at a low humidity.

Fully developed larvae can survive imprisonment within the egg-shell for about 3 hr. at 37° C.

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OBSERVATIONS ON THE DEVELOPMENT OF *LUCILIA SERICATA* (MG.) EGGS IN SHEEP FLEECES

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(With Seven Text-figures)

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INTRODUCTION

Davies & Hobson (1935), making use of the natural hygroscopic properties of wool, measured the relative humidity of the atmosphere within the fleece. They studied a variety of fleece types under various weather conditions, and found that the R.H., except on the rump and anal regions, rarely exceeded 70 % even during wet weather. These results, considered in conjunction with the results of laboratory experiments by Evans (1934) led them to conclude that low humidity in the fleece was the deciding factor in determining susceptibility to blowfly strike, and that the humid conditions in soiled wool around the breech, accounted for the frequency of strike in that region. Evidence confirming these conclusions was obtained by Macleod (1940), in a study of the water balance of the fleece. He showed that the fleece R.H. near the skin under summer conditions rarely exceeded 50 %, and, in the sheep he used, rarely became saturated even after heavy rain.

Recent laboratory studies (Davies, 1948) have shown that *Lucilia sericata* (Mg.) eggs can survive fairly long periods under rather dry conditions at the temperature found near the sheep's skin, and would thus be expected to complete their development in fleeces if rapid increases in R.H. occurred during their incubation period. The question of fleece R.H.'s was therefore reinvestigated in order to determine whether such fluctuations in R.H. did occur during summer weather. Humidity readings were made by means of cobalt-chloride papers (Solomon, 1945)—a method particularly suitable for this purpose since consecutive readings at particular sites in the fleece could be carried out, and a record of day to day changes in fleece R.H.

obtained, with the minimum of disturbance of fleece conditions. In addition a study of the fate of egg batches placed in the fleece was made in order to see how far laboratory results paralleled the behaviour of eggs under field conditions.

I. HUMIDITY CONDITIONS IN THE NATURAL ENVIRONMENT

(*a*) *Method*

Cobalt-chloride papers were used to measure the R.H. of the atmosphere within the sheep's fleece. Papers for use in the field and standards for comparison were prepared according to the method described by Solomon (1945). Pieces of cobalt-chloride paper, numbered before use, of various sizes from 1 × 1 to 1 × 4 cm. were used, and carried to the sheep in dry stoppered tubes. They were carefully inserted end-ways into the fleece, so that one end of the paper rested against the skin and the paper lay along the wool fibres. The position was marked by loosely tying the tips of the wool staples with string. Care was taken to cause as little disturbance as possible to the fleece structure. Standard papers were prepared at 30° C. over a range of R.H. of 30–90 %, at intervals of 5 %. They were made by exposure to the particular humidity for 30 min., and those used in field determinations of fleece humidity were left in position for the same period. On removal from the sheep they were quickly transferred with forceps to a bottle of medicinal paraffin, which preserved the colour they had attained in the fleece. Comparison with standards was made in the laboratory under constant conditions usually within 4 hr. of removing the papers from the fleece.

The most accurate readings were found to be given from 40 to 70 % R.H., where the method was probably accurate to within 3 % R.H. At R.H.'s of 30–40 % no estimate of the precise humidity was made but papers giving such readings were classed below 40 % and are represented on figures as 35 % R.H. When papers were exposed to air well below 40 % R.H. (e.g. 15 % R.H.), their intense blue colour could be distinguished from that at the 30–40 % range. Papers giving this intense blue were thus classified as 'below 30'. Such readings were encountered under winter conditions (Table 2 (*b*)). At humidities of over 70 % R.H., owing to small colour differences, readings could only be made to the nearest 5 % R.H.

In view of the fact that steep humidity gradients were found in the fleece, laboratory experiments were carried out on the effects of humidity gradients on strips of cobalt-chloride paper similar to those used in the field. Conditions on sheep wet with rain were simulated by sticking a large piece of fleece, 5 cm. thick, on to a cardboard sheet. The base of the fleece next to the cardboard was maintained dry by packing it with silica gel, while the free wool tips were maintained moist by spraying with water. In this way a steep R.H. gradient in the fleece atmosphere, of below 40 % at the base, to almost 100 % at the wool tips, was maintained. Cobalt-chloride strips measuring 1 × 3 cm. were placed in this gradient, and the readings at the ends of each paper recorded after various intervals. It was found that very little 'creep' of water occurred within 30 min., the time employed in field readings. After 80 min. appreciable 'creep' occurred, but it was insufficient to affect the colour at the extreme 'dry' end of the strips.

The positions at which papers were placed in fleeces were standardized as much as possible, and were: withers; midback; tailhead; right and left crutch; right and left flank (the flank position being half-way down the sheep's flank and some distance behind the last rib); right and left belly (8 cm. to either side of the umbilicus respectively). Papers were placed at other positions in fleeces, where such readings were likely to be of interest.

In the course of the work, readings by cobalt-chloride papers were obtained for twenty-eight sheep. Daily readings over periods of several days were obtained for nine sheep—five at Houghall Farm, Durham, and four at Crag Farm, Ravenglass, Cumberland. In Table 1 details of five of these sheep referred to in Figs. 2–6 are given.

Table 1. *Details of sheep referred to in Figs. 2–7*

Sheep no.	Breed	Age in months	Sex	Type of fleece
1	'Mule' × Suffolk lamb	6–7	Female	Close compact fleece, of 'Suffolk' type, with fine wiry wool. Length of staple about 8 cm.
2	Swaledale × Border Leicester lamb	3–4	Female	Short fleece, close in comparison with sheep 3, but very open compared with sheep 1. No shed in the wool along the back. Wool falls into small crimpled locks. Staple length about 6 cm.
3	Swaledale × Border Leicester lamb	3–4	Female	Very slack fleece, long coarse fibres, with a marked shed in the wool along the back
4	Swaledale × Herdwick	18	Wether	Fleece entirely brown, short Fairly close, comparatively fine wool
5	Herdwick (Some Swale blood)	18	Wether	Fleece shorter than sheep 4, fairly close. Wool fibres coarse and stiff
7	'Half-bred' × Oxford lamb	6–7	Male	Close fine fleece. Wool less wiry than in sheep 1. Length of staple about 8 cm.

(*b*) *Comparison of the method with those previously used*

A series of parallel fleece humidity readings, using cobalt-chloride papers and the wool weighing technique of Davies & Hobson (1935), were made on three sheep under summer conditions. A further series of triple comparative readings using the present method, the wool method, and the paper hygrometer technique as used by Macleod (1940), were made on three sheep under winter conditions, in December. Care was taken to adhere to the technical details of the methods as given by the above authors, and the results obtained are given in Table 2 (*a*) and (*b*). They show several points of interest. The basal wool sample weighings in Table 2 (*a*) show that their water content was higher than would be expected on the basis of the fleece atmosphere R.H. readings by cobalt-chloride papers; the basal wool, it seems, was not in equilibrium with the basal fleece atmosphere. Table 2 (*b*) shows that this condition also existed in winter. This phenomenon was found also by Macleod when he compared the results of wool calibrations with those of paper hygrometers. He suggested that the positive discrepancy in the results of the wool weighing technique was probably due to continuous skin gland secretion (possibly yolk), which would be expected to be less marked in winter. However this may be, it seems that the wool weighing technique is subject to such positive errors that it cannot legitimately be used to assess the suitability of fleece humidity conditions for blowfly strike near the skin.

Table 2 (*a*). *Comparative humidity readings* (*summer conditions*)

Wool weighing method (samples of basal 2 cm.) (% R.H.)	Readings by cobalt-chloride papers (% R.H.)				Remarks
	At time of sampling		6–12 hr. before sampling		
	Skin surface	2 cm. off skin	Skin surface	2 cm. off skin	
100*	100	100	90+	100	Wool samples taken during long rainy period, when sheep were continuously wet
86	50	70	50	70	
91	—	—	100	100	
74	—	—	51	51	
100*	100	100	100	100	Samples taken 1 day after preceding set. Sheep still wet
78	67	75	70	72	
97*	100	100	100	100	
74	56	67	60	72	
75	56	63	50	65	Samples taken 5 hr. after preceding set. Rain ceased and sheep almost dry
79	65	68	75	75	
86	55	75	58	75	
76	55	65	55	67	
65	40	45	56	63	Samples taken 24 hr. after preceding set. Dry weather
64	42	42	—	—	
70	43	43	55	65	
62	46	46	—	—	Samples taken 3 days after preceding set. Sheep dry. Weather in intervening period dry
50	Below 40	40	—	—	
50	42	45	—	—	

* Samples contained free water. Rain had penetrated to basal 2 cm. of the fleece.

Table 2 (*b*). *Comparative humidity readings* (*winter conditions*) (% R.H.)

Sheep no.	Cobalt-chloride paper method	Paper hygrometer method (Macleod, 1940)	Wool sample method (Davies & Hobson, 1935)
1	Below 30 (30)	19	50
1	Below 30 (40)	26	48
2	40 (58)	50	67
2	Below 30 (42)	21	55
2	Below 30 (40)	22	—
3	42 (42)	40	50
1	Below 30 (40)	16	—

The numbers in brackets refer to the readings 3 cm. off the skin.

In the present paper it will be shown that considerable humidity changes can occur in the basal fleece atmosphere within periods as short as 1 hr.; therefore the time taken by pieces of fleece to regain equilibrium with the fleece atmosphere after a sudden humidity change is of practical importance. In the laboratory, it was found that unwashed naked fleece samples, about 400 mg. in weight, transferred from 0 to 95 % R.H. at 37° C., needed well over 3 hr. to regain complete equilibrium with the higher humidity, while cobalt-chloride strips showed that the air entangled in the fleece samples reached the new humidity (95 % R.H.) within 1 hr. This considerable lag would invalidate results by the wool calibration method during showery weather, when the basal fleece humidities may fluctuate rapidly.

The consecutive humidity readings in Table 2 (*a*) show that rain on the outside of the fleece, although not penetrating to the basal 1 cm., increased the moisture content of the basal fleece during the wet period. This phenomenon was also described by Macleod in his paper and he suggested the possibility of some vapour diffusion process at work along the length of the wool fibres.

Although cobalt-chloride papers cannot be used with accuracy below 40 % R.H., Table 2 (*b*) shows fairly close agreement between R.H. measurements by this method and those by paper hygrometers, at identical sites in the fleeces. Cobalt-chloride papers have distinct advantages over paper hygrometers for fleece humidity measurements in that they are far smaller and can be inserted into the fleece with less disturbance of fleece conditions. Further they are capable of registering the type of humidity gradients which occur in fleeces under British conditions. For example, in Table 2 (*b*), it will be seen in the third set of readings (sheep 2) that cobalt-chloride papers indicated an R.H. gradient in the fleece atmosphere, rising sharply from 40 % near the skin to 58 % R.H. at 3 cm. off the skin. A paper hygrometer placed as close as possible to the skin at this point in a natural shed of the fleece, failed to give any indication of this gradient but showed an intermediate R.H.—namely 50 %. It will be appreciated that a paper hygrometer in the form of a cylinder 1·25 cm. in diameter and 8 cm. long, placed with the long axis parallel to the skin, cannot easily rest in contact (with the latter) along the whole of its length in a fairly close fleece.

(*c*) *Fleece humidity fluctuations, as measured by cobalt-chloride papers*

In the following pages, unless otherwise stated, the terms 'fleece R.H.' and 'basal fleece R.H.' refer to the R.H. of the fleece atmosphere within 1 cm. of the skin of the sheep. The figures except Fig. 6, refer to the R.H. at this position.

Sheep nos. 1–3 and 7, the fleece R.H. readings of which are graphed in Figs. 3–6, were studied at Houghall Farm, Durham, and should be considered in the light of meteorological data taken at this farm and summarized in Fig. 1. Sheep nos. 4 and 5 were at Crag Farm, Cumberland.

The time of day at which particular fleece readings were made is given as G.M.T.

Sheep 4 *and* 5 (Fig. 2). Observations on these sheep were made during cool summer weather. It will be seen that the fleece R.H. near the skin at the midback of sheep 4 was below 40 % during most of the period; this is shown in Fig. 2 as 35 %. It will be seen that when these sheep were wet after rain (e.g. 6, 8 Aug.) the fleece R.H. was appreciably raised, especially in sheep 5, in which after heavy rain it frequently reached 60–80 %. The fleece of this sheep (5)—a Herdwick wether—was short and coarse (see Table 1) and penetration of rain drops to the

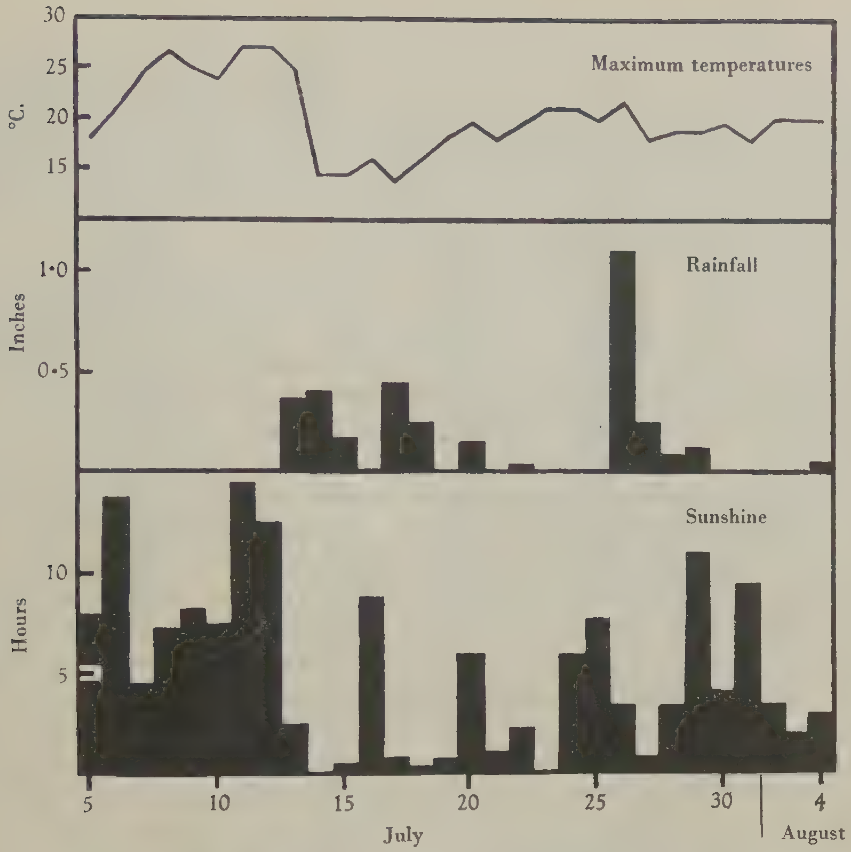

Fig. 1. Meteorological data, Houghall Farm, Durham.

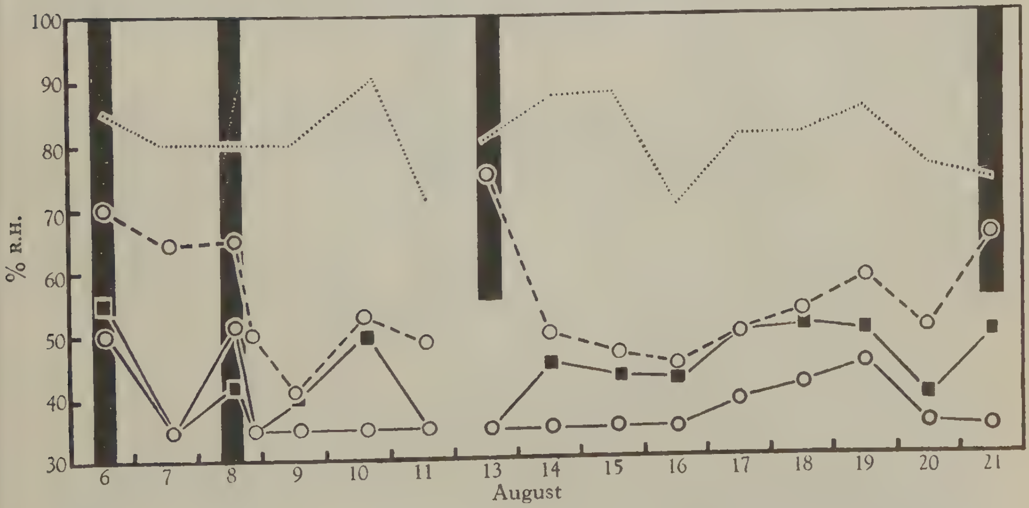

Fig. 2. Fleece atmosphere R.H. variations in sheep 4 and 5. Black columns indicate when the particular sheep was wet with rain, in this and subsequent figures. Sheep 4 ———; sheep 5 - - - - - -; macroclimatic R.H. (read by a sling psychrometer at the same times as the fleece readings were taken) ··················; ○, midback position; ■, right crutch position.

basal cm. of the fleece along the back and flanks was the rule rather than the exception. That the effect of rain on the basal fleece R.H. was of short duration is shown on 8 Aug. where two sets of readings were made—one at 09.00 hr. when the sheep were wet after night rain, and another at 14.30 hr. when the sheep had dried. The readings showed that after the rain dried from the outsides of the fleeces, the R.H. near the skin had fallen rapidly in all cases.

The general picture of fleece humidities in sheep 4 (Fig. 2) is one of low R.H. raised for short periods by the effect of rain and dew, and agrees with the account given by Macleod (1940), who used mainly Down Cross lambs. The same figure, however, shows other features. In sheep 4 the R.H. near the skin at the right crutch was, during most of the period, significantly higher than over the rest of the body, although the fleece was not contaminated with any dung or urine. By estimating the percentage development of freshly laid *L. sericata* egg batches, placed in various parts of the fleece of this sheep, it was shown that only in the crutch was the humidity suitable for blowfly egg development; other parts were too dry (see Section II of this paper). This higher R.H. in the crutch region seems to have been due to more rapid skin secretory activity in that region compared with the rest of the body surface, since cobalt-chloride strips in the former position frequently showed a higher R.H. at the skin surface than at 2 cm. up the wool staple. Other experiments on sheep 4 also pointed to the same conclusion (Cragg & Davies, 1947).

Comparing fleece R.H. readings for sheep 4 with those for sheep 5 (Fig. 2) it will be seen that the fleece of the latter sheep was consistently more humid. In sheep 5, only 37 % of the ninety-three readings taken were at 50 % R.H. or below that level, compared with 79 % in sheep 4 (ninety-four readings). These differences may be due to greater skin secretory activity in sheep 5. This view was confirmed by the fact that in this sheep, when the fleece was not wet with rain, the R.H. was frequently higher near the skin surface than at points farther out in the fleece; this occurred less frequently in sheep 4.

Sheep 2 *and* 3 (Fig. 3). These were two short-fleeced Swaledale × Border Leicester ('Mule') lambs. Humidities are again seen to be below 40 % R.H. when the sheep were dry, but rain on the outside of their fleeces raised the basal humidities considerably, e.g. 22 July. Since their fleeces were short, rain was frequently found to penetrate to the basal cm. of the fleece, and sometimes to the skin along their backs. On their flanks, the wool locks formed an efficient 'roof-tile' system whereby the rain was turned off their bodies. These two lambs, however, did show idiosyncrasies in fleece R.H., possibly correlated with differences in their fleeces (see Table 1). After rain, the fleece R.H. tended to be higher in the close-fleeced sheep 2 than in the slack-fleeced sheep 3 (Fig. 3: 22, 29, 30 July). Macleod (1940) claims to have shown that rain penetrates farther into a close than into a slack fleece. It was noticed in the field that the slack fleece of sheep 3, after rain had ceased, always became dry to the touch in a shorter time than did that of sheep 2. These observations tend to support the contention of Macleod. It is also to be expected that drying processes within the thickness of the fleece would be more rapid in a slack fleece.

Fig. 3 indicates that the fleece humidities of these short-fleeced lambs tended to follow fluctuations of macroclimatic R.H., apart from the direct effect of rain drops in their fleeces.

Suggestion of a diurnal rhythm of fleece R.H., which would be well developed in hot weather with heavy dew or rain during the night, is afforded by the data in

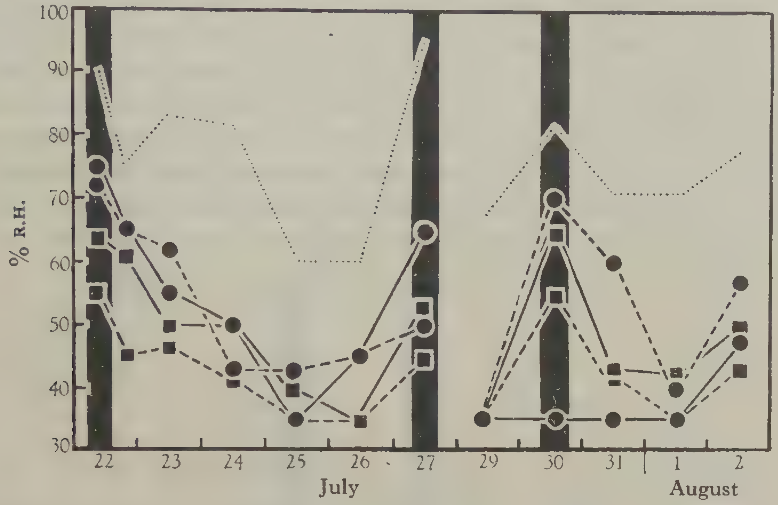

Fig. 3. Fleece atmosphere R.H. variations in sheep 2 and 3. Sheep 2 ———; sheep 3 - - - - -; ●, withers; other symbols as in Fig. 2.

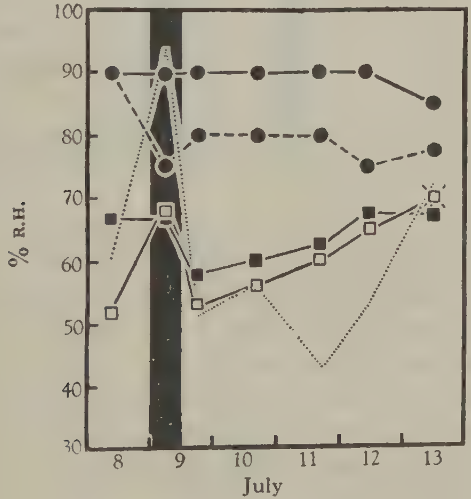

Fig. 4. Fleece atmosphere R.H. variations in sheep 1 and 7. □, tailhead position; other symbols as in preceding figures.

Fig. 3 (22 July) when humidity readings were taken in the morning, after night rain, and again in late afternoon (cf. Fig. 2: 8 Aug., when a similar result was obtained).

Sheep 1 *and* 7 (Fig. 4). The picture of fleece R.H. in these two Down Cross lambs differs markedly from that of the sheep hitherto considered. Humidities in the fleeces of these sheep from 8 to 16 July were above 60 % and frequently above 85 % R.H. For the most part high R.H.'s were confined to the basal 1 cm. or so next to the skin, and cobalt-chloride paper strips indicated very steep humidity gradients, from 60 to 90 % R.H. near the skin, to below 40 % R.H. 3 cm. up the wool staples. To confirm the existence of a high humidity near the skin, on 13 July a batch of laboratory laid *L. sericata* eggs, which had been incubated to within half an hour of hatching, was placed in the fleece, at the withers of sheep 1. Half of the batch was placed at the base of the fleece against the skin, and the other 3 cm. away from the skin. The eggs placed near the skin hatched immediately, and a strike was established at that spot without any addition of moisture; those eggs 3 cm. from the skin did *not* hatch. This shows that the humidity near the skin was at least 80 % R.H., and nearer the outer surface of the fleece very much lower (see Cragg & Davies, 1947). These two lambs (sheep 1 and 7) were healthy and undipped. Drops of yellow-stained watery material were observed on wool fibres near the skin, and were wet to the touch. When cobalt-chloride strips came into contact with them, they produced a characteristic decolorization in which the cobalt salt was washed up the paper, leaving a colourless edge. This phenomenon was similar to that produced by contact with water droplets. Contact with greasy material, causing a translucent stain on the papers, was much rarer.

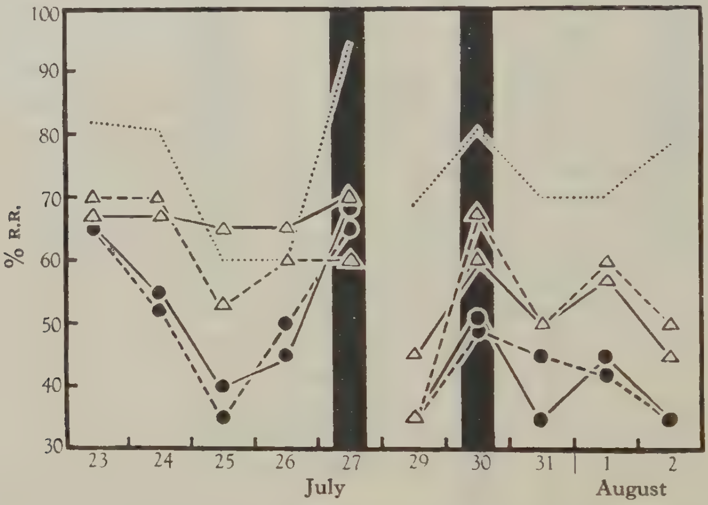

Fig. 5. Fleece atmosphere variations at a later period in sheep 1 and 7. △, right flank position; other symbols as in preceding figures.

Since sheep 1 and 7 were sweating to an extent sufficient to maintain suitable humidities for the development and survival of blowfly eggs and young larvae during the period 8–16 July (Fig. 4), the limiting factor for strike on these sheep must have been that they did not attract blowflies to oviposit on them. The weather during that week was exceptionally hot and dry (Fig. 1). Sweating, as measured by a high humidity close to the skin, was particularly rapid in the withers region in both sheep (Fig. 4), and it appeared to spread to other parts of the body as the hot weather continued. Observations on five other Suffolk Cross and Oxford Cross lambs during the same period showed that similar high fleece R.H.'s existed in four of them, at one or more of the positions where humidity readings were taken.

From Fig. 1 it will be seen that the weather after 13 July became cooler and wetter. Sheep 1 and 7 were dipped in a D.D.T. emulsion on the morning of 18 July and daily fleece readings recommenced on 23 July; some of the results are graphed in Fig. 5. Comparing Figs. 4 and 5, it will be seen that in the second period their fleeces were considerably drier, and quite in keeping with Figs. 2 and 3. In both sheep, however, during the period 23–27 July (Fig. 5), humidities were still high in the flank position. It is concluded that sweating had diminished over most of the body surface, but was still sufficiently rapid on the flanks to maintain high R.H.'s close to the skin. Additional daily humidity readings taken in dry weather showed that rapid sweating frequently occurred over large areas of the flanks of both sheep at this time. Fig. 6 shows that these flank sweat-areas were not constant, but varied from day to day.

Some indication of the relationship of fleece atmosphere R.H. to macroclimatic R.H. is given in Fig. 7, where humidity readings at the skin surface are compared with those at 3 cm. off the skin, and with the macroclimatic R.H., read by a sling psychrometer at the time the fleece readings were taken. The fleece readings refer to the right flank position of sheep 1 during a period when sweating was occurring in that region. From the data in Fig. 7 the following will be noted:

(1) Quite apart from the direct effect of rain, the fleece R.H. at 3 cm. off the skin tended to follow variations in macroclimatic R.H. (e.g. 31 July–2 Aug.).

(2) The skin surface R.H. bore no relation to macroclimatic R.H.

(3) During rapid sweating the fleece R.H. was found to be highest close to the skin: this represents a reversal of the gradient to be expected on grounds of thermal stratification of the fleece atmosphere (e.g. 24–26 July, 31 July–1 Aug.).

(4) The R.H. gradient, due to sweating, may be abolished (27 July) or its direction may be reversed (30 July) by heavy rain.

It was shown by Cragg & Davies (1947) that increased sweating can be produced, in some sheep, by causing them to run. In four such running experiments on one sheep, sweating was detected at the withers in each case, but never at the midback. Since the withers and midback positions were only about 30 cm. apart, sweating would thus appear to be sometimes sharply restricted to particular parts of the body surface. This fact may account for some strikes on clean wool, otherwise difficult to explain. Examples of such strikes, occurring at the same spot on one sheep in successive years, are quoted by Macleod (1943). The following observations show that factors other than muscular exertion also influence the rate of sweating. Fleece humidity readings were taken on sheep 10, a Swaledale Ram lamb, at 09.30 hr. on 20 Aug.; these readings are given in Table 3. The higher humidities next the skin indicate that rapid sweating was occurring, although the sheep had not been running nor was it excited abnormally during the period of the observations. Readings at the same positions on this sheep were carried out 3 hr. later under similar weather conditions. They showed that the fleece atmosphere throughout the thickness of the

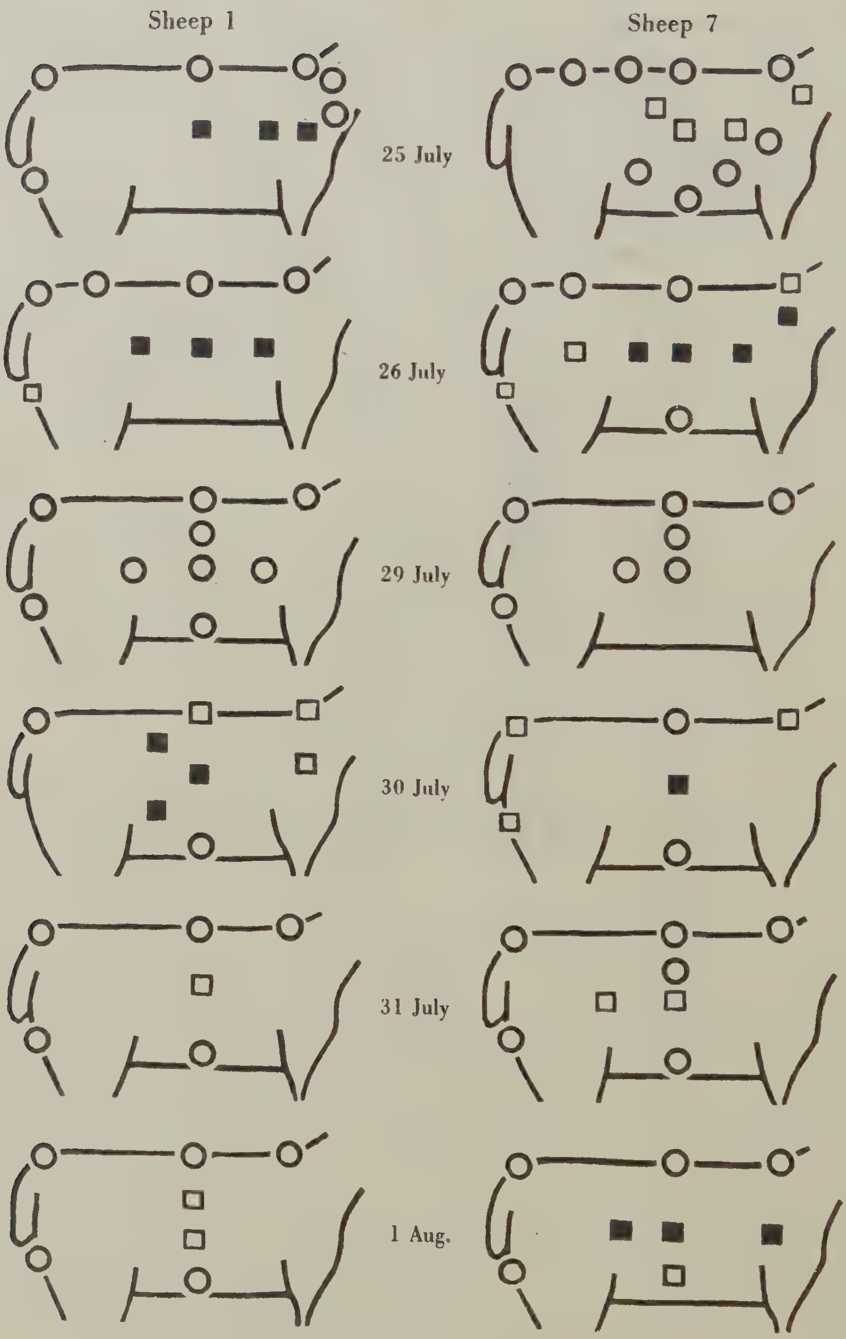

Fig. 6. Fleece humidity readings, sheep 1 and 7. ○, below 50% R.H.; □, 50–59% R.H.; ■, over 60% R.H.

fleece was below 40 % R.H., indicating that sweating had ceased, or at least was proceeding at a slower rate. Simultaneous observations on sheep 4 showed no such variations, although both sheep were kept together and treated identically. It is evident that skin secretory activity in sheep may be governed by factors, other than muscular activity, which are not directly dependent on external conditions such as atmospheric humidity or air temperature.

The above observations also show that moisture at the base of even a thick, close fleece, is very rapidly evaporated, and that to maintain high humidities at its base, suitable for blowfly eggs to develop, a continuous supply of moisture is needed.

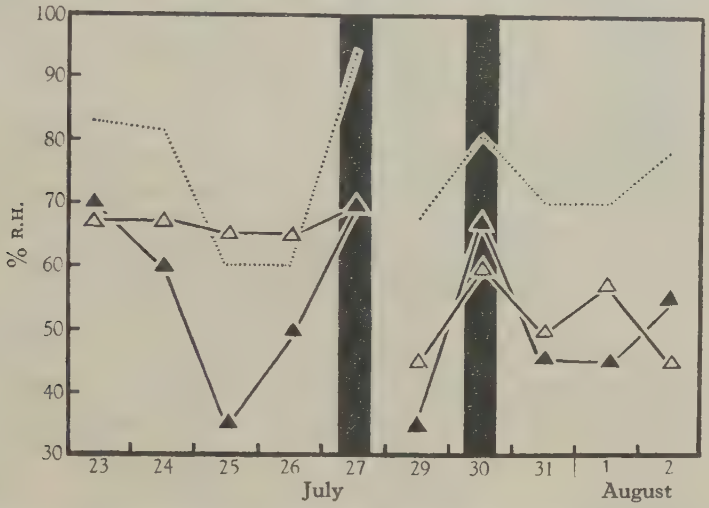

Fig. 7. Effect of sweating and macroclimatic R.H. on humidity within the fleece. △, right flank position, at skin surface; ▲, right flank position, 3 cm. off the skin; ------------- macroclimatic R.H.

Table 3. *Sheep* 10. % R.H. *at* 09.30 hr., 20 August

	Position in fleece				
	Withers	Midback	Tailhead	Right crutch	Right belly
Skin surface	62	60	51	50	Below 40
3 cm. off the skin	Below 40	Below 40	Below 40	50	45

II. THE FATE OF FRESHLY LAID EGG BATCHES IN SHEEP FLEECES

Egg batches, freshly laid by laboratory bred flies, were placed in various positions on the sheep mentioned in the preceding section. A small number of eggs of each batch were set aside in saturated air as controls. Batches were removed from the sheep after 12–16 hr., the constituent eggs teased out after wetting, and the proportion of eggs containing fully developed first instar larvae estimated (Table 4).

The humidity readings in Table 4 (*b*), col. 4, were taken 6–12 hr. after the end of the incubation period of the eggs in question. Thus, they do not necessarily represent the humidity conditions to which the eggs were subjected during development.

Table 4. *Development of eggs in fleeces*

Eggs were placed on the skin of the sheep unless otherwise stated.

(*a*) *Sheep at Houghall Farm, Durham*

Sheep no.	Position of eggs	Cobalt-chloride paper readings at site of egg placement (% R.H.)		Estimated percentage of eggs completing development
		Readings taken 1–5 hr. before eggs were placed in the fleece	Readings taken at time eggs were removed from the fleece	
2	Withers	Below 40	48	> 50
	Withers	Below 40	47	0
3	Withers	60	55	> 50
	Tailhead	Below 40	Below 40	< 50
	Withers	45	56	100
	Tailhead	Below 40	Below 40	< 50
1	Rump	Below 40	—	0
6	Right thigh	Below 40	—	0
	Rump	Below 40	—	0
7	Right flank	51	60	< 50
	Right flank	62	50	< 50
	Right flank	Below 40	55	< 50

(*b*) *Sheep at Crag Farm, Ravenglass*

Sheep no.	Position of eggs	Cobalt-chloride paper readings at site of egg placement (% R.H.)			Estimated percentage of eggs completing development
		Readings taken at time eggs were placed in the fleece		Readings taken 6–12 hr. after end of incubation period	
4	Withers	Below 40		Below 40	0
	Withers	Below 40		Below 40	< 50
	Midback	45		Below 40	0
	Midback	Below 40		Below 40	0
	Midback	70		43	< 50
	Right crutch	50		—	< 50
	Right crutch (4 cm. off skin)	66		—	> 50
	Right crutch	50	50	—	100
	Right crutch	50		50	> 50
5	Right flank	57		63	< 50
	Right crutch	45		57	0
	Withers	75		75	100
	Midback	46		67	0
	Right belly	60		69	100
	Withers	63		—	0
	Withers (3 cm. from skin)	Below 40		—	0
	Withers	66		70	< 50
	Midback	55		68	> 50
8	Left flank	74		75	100
	Right flank	68	68	62	100
	Right flank	55		—	100
	Withers	70		100	0
	Left flank	73		Below 40	> 50
9	Withers	Below 40		60	0
10	Withers	56		61	< 50
	Withers (3 cm. from skin)	Below 40		70	< 50
	Midback (3 cm. from skin)	—		64	100
	Right flank	57		50	100
	Right crutch	50	50	—	100
	Withers	61	Below 40	—	0

In general, the results in Table 4 confirmed laboratory experiments on the *L. sericata* egg, and emphasized the reliability of readings of fleece humidity by cobalt-chloride papers. Using the latter technique, it was shown for sheep 4, that humidities in the crutch region were higher than in most other parts of the fleece (Fig. 2). The humidity in the crutch often approached 50 % R.H.—the minimum humidity for the successful development of eggs at 37° C. These results were confirmed by placing eggs in various parts of the fleece of this sheep (Table 4 (*b*)). Of five batches placed in the midback and withers regions, in three, all eggs failed to complete development, whilst in the other two only 10 % reached the prehatching stage. Of four batches placed in the right crutch, three showed practically 100 % completion of development, whilst 20 % of the remaining 4th batch reached the same stage. Thus only the crutch region was sufficiently humid for the development of *L. sericata* eggs.

Altogether forty-two egg batches were placed in the fleeces of nine sheep; in sixteen batches more than 50 % of the eggs completed development; in twelve between 1 and 50 % of the eggs reached the same stage; whilst in the remaining fourteen batches all eggs died through desiccation at an early stage in development. In a few batches, several eggs hatched whilst in the fleece. Of the batches in which more than 50 % of the eggs completed development, some were taken out of the fleece after 10–12 hr. and placed in saturated air; from these many larvae hatched within a few minutes.

It is clearly evident from the above experiments, that humidity conditions in sheep fleeces are sometimes maintained at a level sufficient for blowfly eggs to complete development. Humidities high enough for hatching, however, occur less frequently.

DISCUSSION

Since a proportion of *L. sericata* eggs can complete development at 50 % R.H. 37° C. (Davies, 1948), the present observations show that suitable humidities for the development of blowfly eggs occur comparatively frequently in the fleeces of some sheep. In such sheep the crucial factor would appear to be the extent and rapidity of rises in fleece R.H. which enable hatching to occur. The rise in basal fleece humidity caused in some way by the presence of free water on the outside of the fleece, described by Macleod (1940), was confirmed in the present investigation. When the outside of the fleece was wet, basal fleece humidities frequently rose to 60–80 % R.H., when some hatching would occur. The fleece atmosphere near the skin did not, however, become saturated, again confirming Macleod's findings. Rain drops were observed to penetrate within 1 cm. of the skin along the back, in short-fleeced Swaledale × Border Leicester lambs, and also in short coarse fleeced Herdwick and Swaledale wethers.

It can be visualized that sudden heavy rain, or sunny showery weather, in addition to promoting fly activity, could provide sudden rises in fleece R.H. and enable eggs to hatch. Under such conditions, the ability of the larvae to remain imprisoned in the shell for 3 hr. (Davies, 1948), is of potential importance, since, especially in hill districts, heavy rain may occur within short periods during weather which is mainly sunny and suitable for fly activity. Blowflies are particularly active during the early forenoon, and eggs laid at such a time would be reaching the hatching condition during a period when dew was falling, or when the sheep might be lying in dew-laden vegetation. The sudden rise in basal fleece R.H. when the outside of the fleece is wet, might lead to hatching of the eggs, especially if they were not laid near the skin. For example, in sheep 1, the basal humidities at the tailhead and right crutch positions at 04.00 hr. on 9 July, a cool morning with very heavy dew, were 68 and 67 % R.H., respectively. At these positions at 14.00 hrs. on the same day when the sheep were dry and the afternoon hot, the humidities had fallen to 53 and 57 % R.H., respectively.

Laboratory experiments (Davies, 1948) have shown that 50 % of *L. sericata* eggs survived in saturated air after exposure for 7–8 hr. to air of 40 % R.H. 37° C., provided the eggs had not previously been exposed to high humidities. Eggs laid under dry fleece conditions in the field would therefore be expected to survive if the humidity rose sharply during the incubation period. The present work showed that such rapid rises in humidity near the skin of sheep, do occur. It seems, however, that the transitory effect of rain in raising the basal fleece R.H. would rarely cause strike to develop, since first instar larvae of *L. sericata* survive for only about 1 hr. at 50 % R.H. 37° C. (Davies & Hobson, 1935).

It was also shown (Davies, 1948) that *L. sericata* eggs, if incubated in saturated air at 37° C. for a few minutes, could withstand very little desiccation when subsequently incubated at a low R.H. This property of the eggs may be of importance in preventing strike development in the field, particularly when eggs are laid immediately after rain, since the water present on the outside of the fleece raises the R.H. near the skin for so short a time.

Both Davies & Hobson (1935) and Macleod (1940) found that the vapour pressure near the skin of sheep, even in dry summer weather, was usually higher than that of the external atmosphere. They considered the excess to be due to evaporation from the skin. It appears from the readings of fleece R.H. given by Macleod (1940) that in the sheep he used, evaporation of water from the skin as measured by the positive balance of vapour pressure near the skin, was much less rapid than was found in some of the sheep used in the present investigation. Among the latter the high R.H. of the basal fleece, due to sweating, showed marked variation in intensity and in distribution over the body. Of twenty-eight sheep of various breeds investigated, five were Down Cross lambs (either Suffolk × Mule or Oxford × Half Bred) of which two, studied in detail, showed continuous sweating in warm weather to such an extent that their fleeces were humid enough near the skin for strike to develop. In these sheep the usual descending R.H. gradient towards the skin (owing to the higher temperature near the latter) was not present. Instead, a gradient in the reverse direction occurred with humidities of 70–90 % R.H. near the skin, whilst the outer layers of the fleece were considerably drier (25–50 %). In none of the other sheep used (various Herdwicks and Swaledales and their crosses) was such continuous general sweating found over large areas of the body. In the latter sheep, as shown in the figures, sweating over more restricted areas and for shorter periods sometimes occurred.

Many farmers consider Down breeds and their crosses to sweat more than other classes of British breeds, especially when fat, and in addition they are considered to be more susceptible to blowfly strike. In one Down Cross lamb (sheep 1, Fig. 4) sweating was found for considerable periods to be more rapid at the withers, a region where strikes occur comparatively frequently (Macleod, 1943).

It appears probable that some part of the suint fraction (Freney, 1940) of the living fleece represents the dried residue of the watery skin secretions which, in the present work, was found at times to raise the R.H. near the skin of some sheep. That rapid changes may occur in the rate of this secretory activity is suggested by R.H. changes which are not referable to weather changes, and which can occur at the base of the fleece (e.g. sheep 10, Table 3 and text). In view of these observations the work of Hobson (1936) appears in a new light. He carried out suint analyses on wool samples from struck and unstruck sheep, and concluded that strike could occur when the suint content was low. This result does not preclude the possibility that susceptibility to strike may sometimes be linked with rapid sweating, leading to high basal fleece humidities. Such activity might render a region suitable for strike development, before any appreciable local rise in the suint content of the fleece could occur. Hobson (1936) also found that in Welsh mountain sheep, flank and belly wool contained more suint than wool from other parts of the body. In sheep studied in the present work, two Down Cross lambs (sheep 1 and 7) showed sweating restricted to the flanks for a considerable period, and one Swaledale Ram, not included in the preceding figures, was also found to sweat continuously on the flanks alone, for a period of 3 weeks. These observations tend to confirm the belief that the suint fraction does have some connexion with the watery skin secretions found in sheep used during the present investigation. Freney (1940) has already suggested that suint may be a constituent of a true sweat, utilized to regulate body temperature.

SUMMARY

1. Comparative determinations of fleece atmosphere R.H. using cobalt-chloride papers and paper hygrometers showed that the former were reliable, although their use was subject to a limited humidity range (40–70 % R.H.). Determinations made by means of wool samples were found to be subject to large positive errors, since the wool near the skin did not appear to be in equilibrium with the fleece atmosphere.

2. Measurements of fleece R.H. by cobalt-chloride papers on twenty-eight sheep of various breeds showed that the R.H. varied considerably from day to day in individual sheep, and in different sheep at the same time. The common condition was one of low R.H., unsuitable for the development of blowfly eggs.

3. R.H. measurements and a study of the fate of *L. sericata* egg batches placed in fleeces showed, however, that conditions suitable for development were occasionally experienced in these sheep. Humidities high enough for hatching were more rarely found.

4. Rapid secretion of watery material by the skin (sweating), leading to suitable humidity conditions for strike, was detected. The various sheep used showed great differences in sweating rates, the most rapid sweating being found in two Down

Cross lambs where, at one period, the limiting factor for strike must have been that the blowflies were not attracted to them.

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